Functional studies of the *otcX3* gene from the *otc* cluster of *Streptomyces rimosus*

By

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List of Abbreviations:

Antibiotic compounds;

ACT	Actinorhodin
ADOT	2-decarboxamide-deoxytetracycline
Ap	Ampicillin
Apra	Apramycin
ATC	Anhydrotetracycline
CDA	Calcium-dependent antibiotic
CTC	Chlortetracycline
DEB	Deoxyerythronolide B
DHK	Dihydrokalafungin
DHOTC	Dehydroxytetracycline
(*S*)-DNPA	4-dihydro-9-hydroxy-l-methyl-10-oxo-3-H-naphtho-[2,3-c]-pyran-3-(S)-acetic acid
FREN	Frenolicin
Gen	Gentamycin
GRA	Granaticin
Kan	Kanamycin
MPT	Methylpretetramid
(*S*)-NHAB	1, 4-naphthoquinone-8-hydroxy-3-[(3*S*)-acetoxy-butyric acid]
OTC	Oxytetracycline
Tc	Tetracycline
TCM	Tetracenomycin
Thio	Thiostrepton

Polyketide/Fatty acid synthase components;

ACP	Acyl Carrier Protein
AT	Acyltransferase
DH	Dehydratase
ER	Enoyl reductase
FAS	Fatty acid synthase
KS	β-ketoacyl synthase
KR	Ketoreductase
PKS	Polyketide synthase
MT	Malonyl transferase
TE	Thioesterase
CLF	Chain length factor
SU	Synthase unit

Others;

ADS	Amplified DNA sequence
AUD	Amplified unit of DNA
CHS	Chalcone synthase

DNA	Deoxyribonucleic acid
Dpg	(*S*)-3, 5, dyhydroxyphenylglycine
FMN	Flavin mononucleotide
FPLC	Flow pressure liquid chromatography
HPLC	High pressure liquid chromatography
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetase
NTG	*N*-methyl-*N*-nitro-*N*-nitrosoguanidine
ONBA	3-Oxo-4-naphthylbutyric acid
ORF	Open reading Frame
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RNA	Ribonucleic acid
RF	Retention factor
RT	Retention time
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAM	(*S*)-adenosyl methionine
SARP	Streptomycete activator receptor protein
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
THN	1, 3, 6, 8-tetrahydroxynaphthalene
TLC	Thin layer chromatography

Abstract

Oxytetracycline (OTC) is a polyketide antibiotic that is made by a Type II polyketide synthase complex. Like such polyketides, OTC is a polymer of acetate extender units. The tetracyclines are unique, however, in that an unusual malonamyl starter unit is used as the priming unit; most other Type II PKS's use an acetate starter.

The genetic architecture of the *otc* cluster includes a putative acyltransferase (AT) gene, *otcX3*. The vast majority of Type II clusters have no AT gene. It has therefore been hypothesized that the product of *otcX3* functions to 'load' the malonamyl starter unit onto the PKS complex. Parallel situations are seen in a variety of other Type II clusters such as a propionyl starter unit for daunorubicin biosynthesis and aklavinone/aclacinomycin in *S. peucetius* and *S. galilaeus*, respectively. As a test of the hypothesis, it was postulated that disruption of *otcX3* would prevent the incorporation of malonamyl into the polyketide backbone. Instead, the closest alternative starter unit, acetoacetyl-CoA, would be used by such mutant. This would then result in the biosynthesis of 2-acetyl-2-decarboxamide-oxytetracycline (ADOT). This compound has severely lower antimicrobial activity compared to OTC.

OtcX3 biosynthesis was tailored by PCR and expressed in *E. coli*. Expression levels after induction were critically dependent on both host strain background and culture conditions. It was planned to express and purify the recombinant protein and assay the affinity to bind malonamyl and other common starter units. However, the recombinant enzyme was unstable and expressed mainly as insoluble inclusion bodies. Purification of soluble recombinant OtcX3 and attempts to refold recombinant OtcX3 from solubilised inclusion bodies were met with limited success. OtcX3 was shown to be dimeric, however, the protein was found to be unstable.

PCR-targeted mutagenesis was used to disrupt *otcX3* on the chromosome of *S. rimosus* M4018; however, complementation of the mutation could not be confirmed. Characterization of the putative mutant strain by agar-plug bioassays, TLC and HPLC analysis confirmed that the putative mutant could still synthesise OTC. This indicated that *otcX3* has a non-essential role in OTC biosynthesis, thus the role of *otcX3* remains unclear.

Chapter 1:
Introduction

1.1 Introduction

The Streptomycetes, of the order Actinomycetales, are among the most important organisms for the pharmaceutical industry. They make a wide range of compounds - from simple amino analogues to aromatic and macrocyclic polyketides. The aim of this thesis was to enhance understanding of polyketide biosynthesis by focussing on the genetics of biosynthesis of the common broad-spectrum antibiotic, oxytetracycline (OTC, Finlay *et al*., 1950) made by *Streptomyces rimosus* (**Fig 1.1**).

The term, 'antibiotics', was introduced in 1943 by Waksman after the identification of antimicrobial metabolites produced by bacteria (Waksman and Schatz, 1943). These first antibiotics had therapeutic action against infectious diseases: streptomycin and neomycin from *Streptomyces griseus* and *Streptomyces fradiae*, respectively (Schatz and Waksman, 1945; Waksman *et al*., 1945). The discovery of streptomycin was of particular significance, as it was the first antibiotic used successfully to treat tuberculosis during mid-period 20th century. Antibiotics produced from *Streptomyces* spp. account for more than half of the antibiotics currently or formerly produced by the pharmaceutical industry since the 1940's, yet the potential for further drug discovery from these organisms still remains highly persuasive (Berdy, 2005; Watve *et al*., 2001; Anderson and Wellington, 2001a; Petkovic *et al*., 2006).

Prior to mid-period 20th century, the importance of antibiotics to public health could not have been foreseen. Despite the importance of antibiotics in fighting infectious diseases, bacterial resistance to antibiotics has developed into a global problem (Neu, 1992). The rapid emergence of multi-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) over the last decade has had massive implications for treatment of such clinical infections. As such, the search for new and effective antibiotics is a major priority. The search for new therapeutic compounds from bacteria will be enhanced through our understanding of the mechanisms used by *Streptomyces* to synthesise polyketides at the biochemical and genetic levels.

1.2 The growth and development of *Streptomyces*

Streptomyces are common saprophytic, Gram-positive, soil-based bacteria that undergo complex morphological and physiological differentiation. In their natural habitat, they survive by colonising and breaking down organic matter in soil using various hydrolytic exoenzymes e.g. proteinases, amylases, ligninases, phosphatases and chitinases (Chater, 1993). The energy generated allows *Streptomyces* to colonise their environment and dominate competing bacteria by synthesising antibiotics (McCarthy and Williams, 1992). *Streptomyces* also produce the neutral oil compound, geosmin, which gives soil its characteristic 'earthy' aroma (Gerber and Lechevalier, 1965; Gust *et al*., 2003).

Although thought to be obligate aerobes, mature development was also observed under anaerobic conditions using standard liquid media (Van Keulen *et al*., 2003). Due to their morphological phenotype, *Streptomyces* were often referred to as fungi and presumed to be eukaryotic. However, their ultrastructure and genetic architecture, studied extensively by Sir David Hopwood using *S. coelicolor* as a model, was found to be characteristic of unicellular bacteria. Therefore, *Streptomyces* are prokaryotic at the genetic level yet exhibit such morphological diversity, making them unique as bacteria (see Hopwood, 1957, 1959, 1965a, 1965b, 1966, 1967, 1969).

Early studies with *S. coelicolor* revealed that growth of the organism was split into distinct 'tissue' phases. After germination of a spore, apical, branching, filamentous growth occurs to form the matted 'bald' vegetative, substrate mycelium. After colonisation of the aqueous environment by the substrate mycelium over 1-2 days, a developmental transition is triggered by complex co-ordination of a range of cellular signals that induces the formation of branching aerial hyphae and ultimately leads to the production and release of exospores, which are dispersed into the surrounding environment (Chater, 1993).

The development of sporulating aerial hyphae gives *Streptomyces* their characteristic 'furry' morphology (Kelemen and Buttner, 1998). The hyphal tips then start to coil, containing many copies of the chromosome. Septation of the hyphae into unigenomic

compartments signals sporulation (Chater, 1998; Kelemen and Buttner, 1998), in a similar way to endospore formation in *Bacillus subtilis* (Dubnau *et al*., 1988). Prior to sporulation, biosynthesis of macromolecular compounds (DNA, proteins and storage compounds e.g. glycogen) is reduced (Granozzi *et al*., 1990). Autolysis of cells in the substrate mycelium provides additional nutrients that are then used for the generation of the aerial mycelium (Wildermuth, 1970). Under laboratory conditions, antibiotic biosynthesis is normally detected during the transition from development of substrate mycelium into sporulating aerial hyphae (Demain, 1998). Maturation of spores is identified by the thickening and rounding of the spore walls combined with pigmentation and arrangement into spore chains that are eventually released, thus 'completing' the life cycle (**Fig 1.2**, Chater and Merrick, 1979).

Most of the research undertaken to improve our understanding of the developmental biology of *Streptomyces* has come from genetic studies of *S. coelicolor* mutants, defective in different stages of the growth cycle. Two classes of mutant were initially identified; white (*whi*) mutants that could form aerial mycelium and synthesise antibiotics but could not form mature, pigmented spores (Hopwood *et al*., 1970; Chater, 1972; McVittie, 1974; Schwedock *et al*., 1997; Flardh *et al*., 1999) and bald (*bld*) mutants that could not produce sporulating, aerial hyphae or synthesise antibiotics (Hopwood, 1967; Merrick, 1976). Ongoing research has improved understanding of the complex network linking *bld*, *whi* mutants and other related genes, leading to a clearer picture of the complex, highly-regulated system that involve these genes (**Fig 1.2**, Chater, 2001).

1.2.1 Key studies related to *whi* mutants in *Streptomyces* colony development

To form septa during spore formation in *S. coelicolor*, several 'early' *whi* genes are necessary: *whiA, B, G, H, I* and *J* (Chater, 1972; Chater, 1993; Chater, 2001). The white phenotype is due to the inability to synthesise the grey spore pigment WhiE, encoded by the Type II PKS (**Section 1.4)** *whiE* locus (Kelemen *et al*., 1998).

WhiG encodes a RNA polymerase sigma factor that plays a significant role in the development of mature spore chains, σ^{WhiG} (Chater *et al*., 1989). Transcription of *whiG* was not affected by any of the early *whi* genes nor was it restricted to the

growth of aerial hyphae, placing *whiG* at the top of the '*whi*' gene hierarchy but leaving the mechanism of its transcriptional activation unclear (Kelemen *et al*., 1996). *WhiA* and *whiB* both have two promoters; one low-level constitutive promoter that is active during vegetative growth and another that is up-regulated during aerial hyphal growth/sporulation and is also independent of σ^{WhiG} activity (Ainsa *et al*., 2000; Soliveri *et al*., 1992, 2000). The σ^{WhiG} RNA polymerase holoenzyme transcribes *whiH* and *whiI* (Ryding *et al*., 1998; Ainsa *et al*., 1999) and is the main link between the *bld* mutants blocked earlier in growth and the other *whi* mutants, since its expression was found to be repressed by the product of *bldD* (discussed below, Elliot *et al*., 2001). Transcription of a later gene encoding an important sigma factor, *sigF*, was found to be dependent on the transcription of *whiG* (Kelemen *et al*., 1996). σ^{F}, which has a homologue in endospore formation of *Bacillus subtilis*, plays a crucial role in the correct maturation of spores. A *sigF* mutant produced irregular, thin-walled, poorly pigmented and detergent-sensitive spores with uncondensed DNA (Potuckova *et al*., 1995). The promoter for *sigF* did not contain any of the known binding sites for the σ^{WhiG} RNA polymerase holoenzyme, indicating an indirect role in the regulation of *sigF*, possibly through the activity of an intermediate protein e.g. WhiH (Kelemen *et al*., 1996).

'Early' *whi* genes are necessary for transcriptional up-regulation of *ftsZ* whose product plays a crucial role in sporulation septation (Flardh *et al*., 2000). A strain mutated in the promoter region for *ftsZ* had a phenotype similar to *whiH*, indicating a possible regulatory role in transcription of *ftsZ* by WhiH (Flardh *et al*., 2000).

Many *bld* mutants have been identified and each display pleiotropic phenotypes. These include *bldA, B, C, D, F, G, H, I, J* (formerly *bld261,* Nodwell *et al*., 1999), *K* (Kelemen and Buttner, 1998), *M* and *N* (formerly *whiK* and *whiN*, Molle and Buttner, 2000; Bibb *et al*., 2000). Most *bld* mutants can be rescued phenotypically, in part, by growth on a poor carbon source such as mannitol, with the exception of *bldB* mutants (Pope *et al*., 1996).

On rich media, most *bld* mutants are unable to produce a small hydrophobic, morphogenic surfactant, lantibiotic-like protein called SapB that is crucial for aerial hyphae formation by breaking the aqueous surface tension of the substrate mycelium,

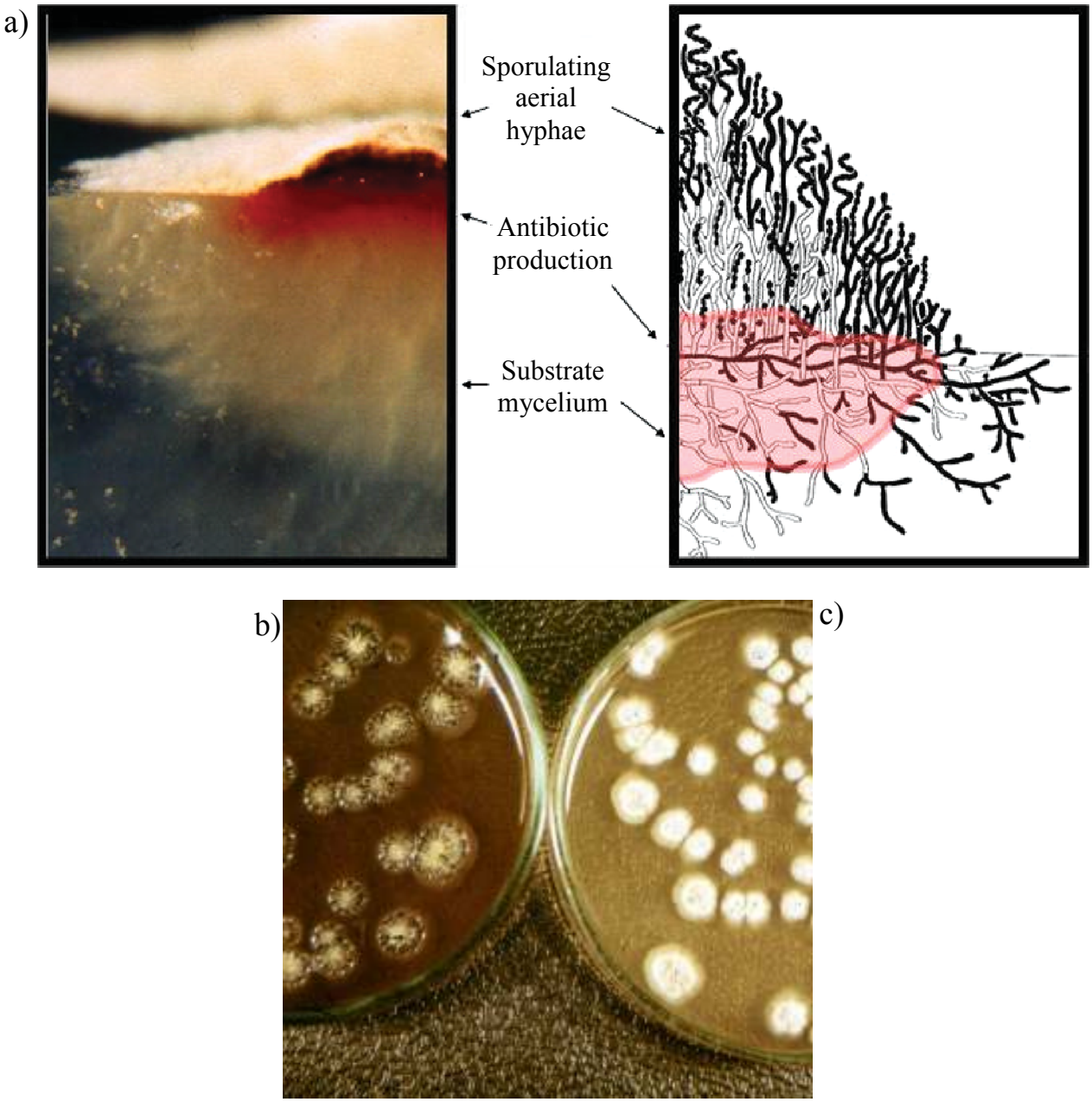


Fig 1.1 Cross section of a *Streptomyces coelicolor* colony and aerial morphology of *S. rimosus* R6 and R7

a) Diagram illustrating separate growth phases of a mature sporulating *S. coelicolor* colony. Active cells indicated by black, older/lysed cells in white (Chater, 2006).

b) *S. rimosus* R6 showing the characteristic 'Kuglof' morphology and reduced sporulation

c) *S. rimosus* R7 (ATCC10970) (Petkovic *et al*., 2006)

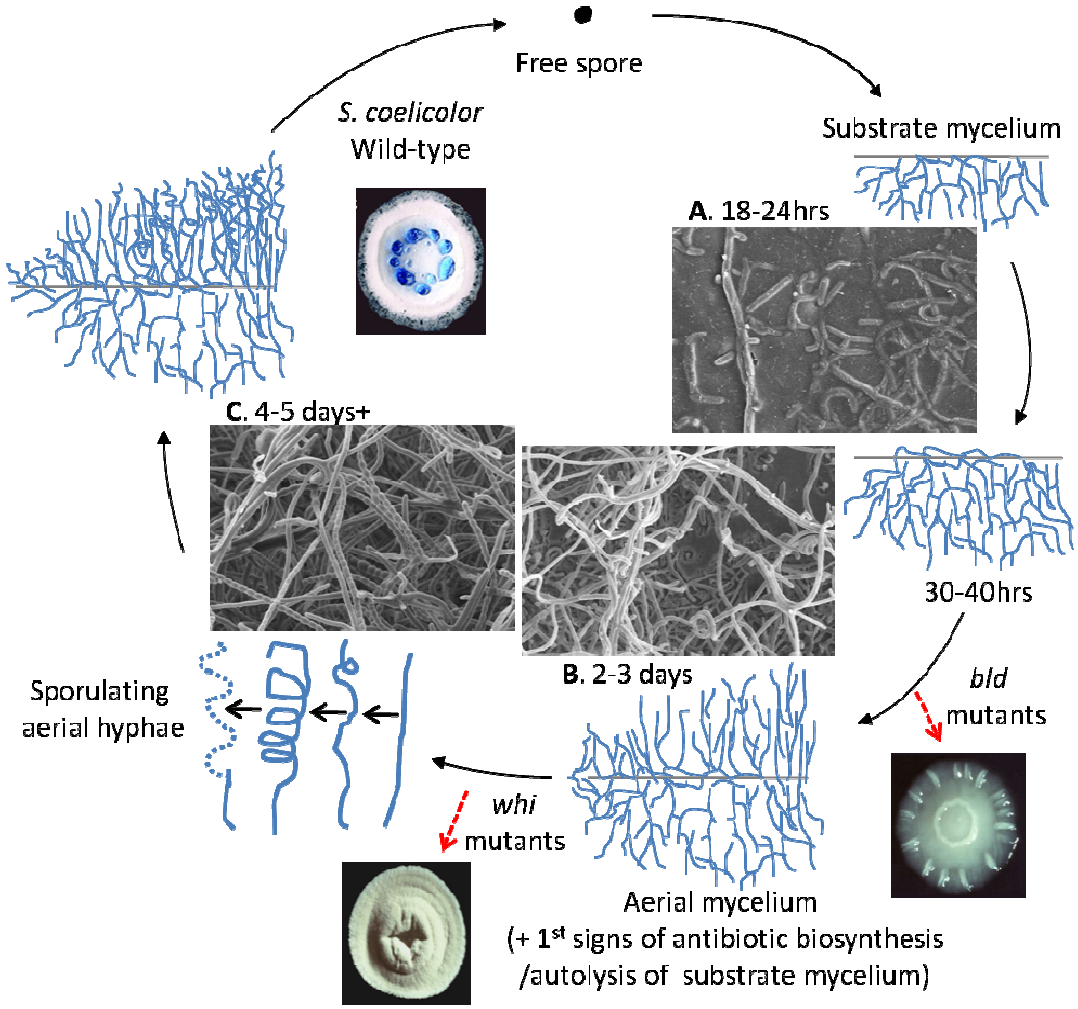


Fig 1.2 General life cycle of a *Streptomyces* colony

Photographs of *S. coelicolor* wild-type colony, *whi* and *bld* mutant used with courtesy from Hoskisson, P. (personal comm.). Photographs of different stages of developing mycelium taken from Claessen *et al*., (2006): **A** – substrate mycelium; **B** – aerial mycelium; **C** – sporulating aerial hyphae. See text for discussion of *bld* and *whi* mutants. Red dashed arrows indicate the general stage of growth at which the various *bld* and *whi* mutants are blocked.

thus enabling the growth of aerial hyphae (Willey *et al*., 1991; Tillotson *et al*., 1998; Kodani *et al*., 2004). When purified recombinant SapB was applied exogenously to *bld* mutants, they regained their ability to form aerial hyphae (minus the ability to sporulate), with the exception of *bldF* (Tillotson *et al*., 1998). SapB is derived post-translationally from RamS, part of the *ram* operon, itself under control by the response-regulator, *ramR* (Kodani *et al*., 2004). When *ramR* (which has a characterized orthologue involved in *B. subtilis* development, *spoOA*) was disrupted, no SapB was detected, leading to stunted aerial hyphal growth (Nguyen *et al*., 2002).

1.2.2 Key studies related to *bld* mutants in *Streptomyces* colony development

A hierarchal cascade of extracellular signals produced by *bld* mutants was proposed by Willey *et al*., (1993) for growth on rich media. The wild-type phenotype could be restored to certain *bld* mutants when grown close to other *bld* mutants that secreted the extracellular signal that the other was unable to produce. Thus, the following cascade was initially proposed, with the higher mutants able to restore the wild-type phenotype in the preceding mutant but not vice versa: *bld261/bldJ* < *bldK* < *bldA,bldH* < *bldG* < *bldC* < *bldD* → SapB (Willey *et al*., 1993; Nodwell *et al*., 1996). Evidence suggested, however, that this cascade was too simplistic to fully describe this potential pathway. *BldB* mutants have a phenotype that suggests that *bldB* should belong in the *bldC* group, yet cannot complement *bldA* or *bldH* mutants. *BldI* appears to belong with the *bldA,H* group yet cannot be complemented by *bldC*. This indicated that either more *bld* mutants had yet to be discovered or that the regulation of transcription of other significant developmental genes was more complex (Willey *et al*., 1993; Nodwell *et al*., 1996).

Another group of proteins important for aerial hyphae formation were characterized recently. Rodlins form a highly stable, insoluble layer of parallel rods as part of the surface of aerial hyphae and spores. When two genes, *rdlA* and *rdlB* were disrupted, this layer was lost but the hydrophobicity of the hyphae was not (Claessen *et al*., 2002). Eight genes were subsequently identified that encoded another family of insoluble structural proteins, named the chaplins. Three short proteins encoded by *chpA, B, C* and five long encoded by *chp D, E, F, G, H* were described (Elliot *et al*., 2003). Chaplins, like SapB, lower the aqueous surface tension of the substrate

hyphae, thus enabling aerial hyphal growth. Chaplins function by assembling into hydrophobic, amyloid-like fibril mosaic layers that coat the aerial hyphae (Claessen *et al*., 2003). Null mutants displayed delayed aerial hyphal growth; however, exogenous application of purified recombinant chaplins restored normal growth to the mutant, indicating Rodlins and Chaplins to be as important as SapB in the erection and stability of aerial hyphae development (Claessen *et al*., 2003). This has led to a revision of the pathway involved in aerial hyphal development and has been referred to as the 'sky' pathway (see Claessen *et al*., 2006 for a review).

The *bldA* mutant, renamed by Merrick (1976) from its original S48 annotation by Hopwood (1967), was the first of the *bld* genes to be characterized. It encodes the corresponding leucyl tRNA (UUA) for the rare codon TTA (Lawlor *et al*., 1987). This codon is rare because of the naturally high %G+C content of streptomycetes. It was initially found only in genes encoding pathway specific-regulatory proteins e.g. *actII-ORF4* (**Section 1.8**, the sustained transcription of which, was shown to be dependent on BldC (Hunt *et al*., 2005) and encodes a protein that directly activates actinorhodin biosynthesis, Fernandez-Moreno *et al*., 1991). It was also found in the putative exporter, *actII-ORF2* (Caballero *et al*., 1991), the (indirect) pathway activator for the red-pigmented antibiotic, undecylprodigiosin *redZ* (Guthrie *et al*., 1998) and also in some resistance and regulatory genes from other *Streptomyces* spp. (Leskiw *et al*., 1991).

Genetic evidence indicated that the TTA codon had not been conserved in any of the essential genes required for vegetative growth or biosynthesis of secondary metabolites and is only found in genes with important regulatory roles (Leskiw *et al*., 1991). Sequencing of the *S. coelicolor* A3(2) genome has shown that only 145 out of 7825 genes contain the TTA codon and none appear to be essential 'housekeeping' genes (Bentley *et al*., 2002), thus indicating the pleiotropic effects of *bldA* over the development and regulation of many cellular processes. TTA codons were subsequently found in 2 genes, *mmfL* and *mmyB,* on the linear plasmid, SCP1 that contains the cluster for the synthesis of the antibiotic, methylenomycin. When point mutations were introduced into these genes to encode the more common leucyl codon, CTC, the *mmfL* gene produced an extracellular compound, interpreted by the product of *mmyB* that allowed the *bldA* mutant to activate methylenomycin

biosynthesis (Bentley *et al*., 2004; O'Rourke *et al*., 2009). For reviews on the direct and indirect influence of the *bldA* regulon, see Chater (2006), Hesketh *et al*., (2007) and Chater and Chandra (2008).

The original *bldA* and *bldH* mutants were very similar in morphological phenotype (Merrick, 1976) and combined in the same complementation group in the extracellular signalling cascade proposed by Willey *et al*., (1991; 1993). The *Sau*3A1 fragment that complemented the original *bldH* mutant contained an ORF with a TTA codon (Takano *et al*., 2003). This gene showed homology to *adpA* (84% identity) from *S. griseus*, which encodes an A-factor-dependent transcriptional activator, responsible for the onset of streptomycin production as part of the signalling cascade generated by the most historically significant γ-butyrolactone, the 'microbial hormone', A-factor (2-isocapryloyl-3R-hydroxymethyl-y-butyrolactone) (Khokhlov *et al*., 1967; Ohnishi *et al*., 1999). When the TTA codon in a *bldH* mutant was engineered to a non-BldA-dependent TTG leucyl codon, aerial mycelial growth was partially restored to a *bldA* mutant provided that the downstream gene, *ornA,* was present in its natural chromosomal arrangement (Takano *et al*., 2003). Therefore, one of the (if not the principle) routes of the *bldA* morphological phenotype in *S. coelicolor* had been characterized.

BldD was isolated on a 1.3kb fragment of DNA from a cosmid library of *S. coelicolor* that complemented the original *bldD* mutant (Merrick, 1976; Elliot *et al*., 1998) and bioinformatic data revealed it to be similar to other DNA-binding proteins (Elliot *et al*., 1998). It was subsequently determined that BldD was a central regulator of development in *S. coelicolor*. Transcriptional studies on the activity of the promoter in the mutant indicated that BldD repressed its own synthesis (Elliot *et al*., 1998). BldD binds to the promoters of the genes encoding the developmentally-important sigma factors *whiG* and *bldN*, as well as a previously uncharacterized gene that encodes another putative transcription factor, *bdtA* (Elliot *et al*., 2001). Transcription of these genes was also shown to be up-regulated during vegetative growth in a *bldD* mutant when compared to the *bldD*$^{+}$ mutant. This indicated that BldD was crucial for the repression of these genes during early growth, thus defining its role as a central regulator of *Streptomyces* development (Elliot *et al*., 2001). The significance of repression of *whiG* by BldD remains unclear. However, *whiG*

Mutant	Description	Reference
whiA	Dual promoters: 2nd not affected by σ^{WhiG}. Function unknown, conserved among Gram-positives.	Ainsa *et al*., (2000) Flardh *et al*., (1999)
whiB	Dual promoters as *whiA*. Prototype of small DNA-binding protein family, conserved in actinomycetes.	Soliveri *et al*., 1992, 2000 Flardh *et al*., (1999)
whiD	Paralogue of *whiB*. Dual promoters. Transcribed later in spore maturation	Molle *et al*., (2000)
whiE	Locus encoding Type II PKS production of grey spore pigment, WhiE	Kelemen *et al*., (1998)
whiG	Important sigma factor. Main link between *whi/bld* mutants. Transcription repressed by BldD	Chater *et al*., (1989) Kelemen *et al*., (1996) Elliot *et al*., (2001)
whiH	Putative GntR-like regulatory protein	Ryding *et al*., (1998)
whiI	Transcribed by σ^{WhiG} holoenzyme. Encodes response-regulator-like protein	Ainsa *et al*., (1999)
whiI	Encodes a small protein belonging to a family exclusive to streptomycetes	Gehring *et al*., (2000)
bldA	Encodes the rare leucine anti-codon $tRNA_{UUA}$	Lawlor *et al*., (1987)
bldB	Small DNA-binding molecule. Function unknown. 24 homologues in *S. coelicolor* genome	Pope *et al*., (1998); Fernandez-Moreno *et al*., (1992b); Sheeler *et al*., (2005)
bldC	Small DNA-binding molecule. Homologues found in Gram-positive and Gram-negative bacteria	Hunt *et al*., (2005)
bldD	Central regulator of development in *S. coelicolor*	Elliot *et al*., 1999; 2001; Kelemen *et al*., (2001)
bldG	Putative anti-sigma factor	Bignell *et al*., (2000)
bldH	AdpA homologue containing TTA codon	Takano *et al*., (2003)
bldI	Unknown	Leskiw and Mah, (1995)
bldJ	Unknown	Nodwell and Losick, (1998)
bldK	Oligopeptide transporter	Nodwell et al., (1996); Nodwell and Losick, (1998)
bldL	Unknown	Nodwell *et al*., (1999)
bldM	Response regulator probably active in early and late stages of development	Bibb et al., (2000); Molle and Buttner, (2000)
bldN	Extracytoplasmic sigma factor; recognizes promoter region of *bldM*, probably active in early and late stages of development	Elliot *et al*., (2001); Bibb *et al*., (2000)

Table 1.1 Summary of *whi* and *bld* mutants in *S. coelicolor*

transcripts are detected throughout vegetative and aerial hyphal growth (Chater, 2001). The mechanism by which BldD repression is 'switched off' during aerial hyphae and sporulation development is also still unclear (Elliot *et al*., 1998; Chater, 2001). For reviews on developmental mutants, see Chater, (2001) and Claessen *et al*., (2006), and the references cited for each mutant in **Table 1.1**.

1.2.3 γ-butyrolactones and ppGpp synthesis in Streptomycetes

In *S. griseus*, A-factor is made by the product of *afsA* using by-products of fatty acid metabolism (Ando *et al*., 1997). A-factor functions by binding to ArpA, a repressor-like receptor protein that binds to the promoter region of the gene *strR*, which encodes the pathway-specific activator for streptomycin (Distler, *et al*., 1992). As the intracellular concentration of A-factor reaches a critical level, derepression caused by ArpA binding to A-factor allows the flexible DNA-binding protein AdpA to bind to a specific sequence upstream from *strR,* thus activating the transcription of *strR* (Ohnishi *et al*., 1999; Ohnishi *et al*., 2005; Tomono *et al*., 2005a). In the same study, an *adpA* mutant failed to develop aerial hyphae indicating a pleiotropic role for AdpA (Ohnishi *et al*., 1999). DNA microarray analysis between the genome of the wild-type and the Δ*adpA* mutant revealed that the transcription of over 1,000 genes was affected directly or indirectly (Ohnishi *et al*., 2008). For articles on selected key developmental genes in *S. griseus*, see (Yamazaki *et al*., 2000; 2003b; Tomono *et al*., 2005b; Kato *et al*., 2002; Chater and Horinouchi, 2003).

In *S. coelicolor*, at least three γ-butyrolactones have been identified, of which SCB1 is the best characterized (Takano *et al*., 2000) (for reviews, see Takano, 2006; Nishida *et al*., 2007). SCB1 is the primary γ-butyrolactone involved in antibiotic regulation as it has been shown to initiate actinorhodin and undecylprodigiosin biosynthesis. It is synthesised by the orthologue of *afsA*, *scbA*, which is itself regulated by the product of *scbR*. Together they form a repressor-amplifier relationship that has been found in the production of other γ-butyrolactones in streptomycetes (Takano *et al*., 2000; 2001). It is likely that the sequencing of the *S. rimosus* genome will reveal homologous hormonal signalling systems that regulate the biosynthesis of oxytetracycline and other cell processes.

Another significant signalling molecule that has global effects on the biology of a growing *Streptomyces* colony is the stringent factor, the highly phosphorylated guanine nucleotide, ppGpp. Levels of ppGpp have been found to have an influence over antibiotic biosynthesis, transcription of conservons and morphogenic proteins (Hesketh *et al*., 2007b). The effects of ppGpp synthesis were first studied in *E. coli* in response to amino acid limitation and were found to down-regulate processes such as rRNA biosynthesis and ribosome production (Sands and Roberts, 1952; Stent and Brenner, 1961) and has been found to act as a global regulator in *E. coli* by binding to RNA polymerase, thus inducing a widespread transcriptional shift (Magnusson *et al*., 2005).

In *S. coelicolor*, ppGpp is synthesised by the product of the gene, *relA* (Chakraburtty and Bibb, 1997). A *relA* null mutant initially produced a bald phenotype that displayed retarded aerial growth with no sporulation (Chakraburtty and Bibb, 1997). Under conditions of limited nitrogen, the *relA* mutant was also deficient in the production of actinorhodin and undecylprodigiosin (Chakraburtty and Bibb, 1997; Sun *et al*., 2001). DNA microarray analysis revealed the global effects of ppGpp. Under certain growth conditions, ppGpp synthesis is required for redirection and co-ordination of gene transcription in *S. coelicolor* to complete its developmental life-cycle (Hesketh *et al*., 2007b). Its synthesis repressed transcription of the major sigma factor of *S. coelicolor*, *hrdB,* as well as many other conserved genes (Buttner *et al*., 1990; Hesketh *et al*., 2007b). Transcription of the pathway activators for calcium-dependent antibiotic and actinorhodin, *cdaR* and *actII-ORF4* were also shown to be induced by ppGpp synthesis (Hesketh *et al*., 2001; 2007b). The *relA* mutant overproduced SapB but could not produce the Rodlins or Chaplins, thus explaining the failure of the mutant to sporulate (Hesketh *et al*., 2007b).

1.3 The *Streptomyces* genome

Streptomyces are generally characterized as having a high percentage of guanine and cytosine in their DNA, averaging about 72% (Gladek and Zakrzewska, 1984). Their chromosome size is often around 8-9Mb with terminal inverted repeats. Characterization of *Streptomyces* chromosomes has shown them to be linear, which is now accepted as being a typical characteristic of the genus. Proteins are covalently

bound to the 5' end of chromosomal DNA and are used as primers for replication (Lin *et al*., 1993).

DNA also exists in *Streptomyces* as high or low copy circular and large linear plasmids (Kinashi *et al*., 1987). Examples of these are the autonomous, complex 350kb linear plasmid, SCP1 (Kirby *et al*., 1975; Wright and Hopwood, 1976) that was sequenced and characterized more recently (Bentley *et al*., 2004) and the 31kb low copy circular plasmid, SCP2 and its mutational variant, SCP2* that plays a major role in the fertility status of *S. coelicolor* A3(2) (Bibb *et al*., 1977) and has also been sequenced (Haug *et al*., 2003). Gene clusters for the biosynthesis of some antibiotics have been found on plasmids in *Streptomyces* such as methylenomycin on SCP1 (Wright and Hopwood, 1976) and for lankacidin and lankamycin biosynthesis amongst others on the linear plasmid pSLA2-L of *S. rochei* (Mochizuki *et al*., 2003). Another large linear plasmid, pPZG103, derived from pPGZ101 as a result of recombination between the chromosome and pPGZ101, was isolated from *Streptomyces rimosus* R6 during studies into chromosome instability and found to contain multiple copies of the *otc* cluster (Pandza *et al*., 1998). However, these represent only a few plasmids carried by *Streptomyces* that actually have characterized phenotypic properties (Bentley *et al*., 2004).

The sequencing of the *S. coelicolor* A3(2) genome was completed in June, 2001 (Bentley *et al*., 2002). This project was a major turning point towards increasing the rate of our understanding of *Streptomyces* biology in general. Four antibiotics were known to be produced by *S. coelicolor* prior to completion of the genome sequence; actinorhodin (ACT), the red-pigmented undecylprodigiosin (Hopwood and Wright, 1983; Lakey *et al*., 1983), calcium-dependent antibiotic (CDA) (Tsao *et al*., 1985) and methylenomycin (Wright and Hopwood, 1976b). However, further clusters for secondary metabolites have been found. Bioinformatic analysis of the *S. coelicolor* genome revealed 18 additional secondary metabolite clusters. These included polyketides synthesized by polyketide synthases of Type I, II and III (**Section 1.4**, Bentley *et al*., 2002). Clusters for the biosynthesis of peptides assembled by non-ribosomal peptide synthases (NRPS), iron-binding siderophores (desferrioxamine), hopanoids, γ-butyrolactones, chalcone, terpene and geosmin were also revealed (Bentley *et al*., 2002; Barona-Gomez *et al*., 2004; Gust *et al*., 2003).

The 9.0Mb genome sequence for the industrially important *S. avermitilis*, producer of avermectin, was published shortly after that of *S. coelicolor* (Ikeda *et al*., 2003), followed most recently by the 8.5Mb genome of *S. griseus* IFO 13350 (Ohnishi *et al*., 2008). Each genome shared many characteristics as well as displaying several unique. A conserved internal core of 6-6.5Mb was found in each genome, with subtelomeric regions of 1-2Mb, now believed to be a common characteristic of *Streptomyces* genomes. Some unique traits of *S. griseus* were its lack of a *whiE* or *bldK* locus, yet bioinformatic evidence suggested that the genome contained alternatives to these developmentally-important loci (Ohnishi *et al*., 2008). Most of the clusters identified for known or putative secondary metabolites in each genome were located outside of the conserved core, another accepted characteristic of the genus; indicating that a large proportion of the metabolic diversity found in *Streptomyces* has resulted from evolutionary development of these sub-telomeric regions. For more detailed analysis of each genome, see Bentley *et al*. (2002), Ikeda *et al*. (2003) and Ohnishi *et al*. (2008).

Research on *S. rimosus* has been published using several different strains. From the late 1950's until the early 1970's, laboratories in Moscow, Russia, at the Instititute of Genetics and Selection of Industrial Microorganisms and the All-Union Research of Antibiotics, carried out research using the strain known as LS-T118 (Mindlin, 1969). In Croatia, at the PLIVA Research institute, the Ruder-Boskovic Institute and the University of Zagreb, research has been published since the early 1960's using two nearly identical strains, according to their linkage maps (Alacevic *et al*., 1973). R6, a soil isolate from the University of Zagreb used for the commercial production of OTC at PLIVA and the strain ATCC 10970 (NRRL 2234) (**Fig 1.2b, c**), often referred to as R7. During the 1970's, Pfizer, in collaboration with the John Innes Centre in the UK, developed and created mutants from two prototrophic strains of *S. rimosus* used for the commercial production of OTC by Pfizer - M4018 (Rhodes *et al*., 1981) and 15883 (Hunter and Hill, 1997; McDowall *et al*., 1999). M4018 has been used in more recent research on *S. rimosus* at the University of Glasgow (Garven, 1995; Petkovic, 1998) and is the strain that was used in this work.

The genetics of the OTC producer *S. rimosus* are among the most understood of the industrially-significant streptomycetes but although in progress, the genome has yet

to be sequenced (Hunter, personal comm.). The first genomic linkage maps were elucidated in the early seventies (Friend and Hopwood, 1971; Alacevic *et al*., 1973), which were expanded upon to produce wider mapping of the chromosome (Alačević *et al.*, 1978; Rhodes *et al*., 1981). DNA renaturation kinetics was used to produce the first estimate of the genome size. This work compared the genomes of *S. coelicolor* A3(2) and *S. rimosus* R7 and found that the two species did not differ significantly in G+C-content (73.0% for *S. coelicolor*, 71.4 % for *S. rimosus*) or size (Benigni *et al*., 1975). Early mapping studies of the *otc* cluster indicated that it was located on the chromosome (Pigac and Alicevic, 1979). A restriction map of the R6 strain using pulsed-field gel electrophoresis (PFGE) was constructed. Analysis using the restriction enzymes *Ase*I and *Dra*I showed that the chromosome was linear, as observed in other streptomycetes and was estimated to be around 8 Mb in size, which corresponded to the true size of 8.6Mb (Gravius *et al*., 1993 Pandza *et al*., 1997).

Genetic instability is not uncommon in bacterial species but occurs more frequently in *Streptomyces* (Birch *et al*., 1990; Cullum *et al*., 1988; Chen, 1995; Volff and Altenbuchner, 1998; Wenner *et al*., 2003) and has been most extensively studied in *S. lividians*, *S. ambofaciens* and *S. rimosus* (Altenbuchner and Cullum, 1984; Leblond *et al*., 1991; Wenner *et al*., 2003; Gravius *et al*., 1993). Early studies on the instability of the *S. fradiae* genome used the terms 'amplifiable unit of DNA' (AUD) and 'amplified DNA sequence' (ADS) to describe a region of DNA that was subject to amplification and the exact sequences, respectively (Fishman and Hershberger, 1983). The ADS often varied but was always located within a specific AUD region. This was described as Type I amplification of which genetic instability in *S. ambofaciens* is one example (Leblond *et al*., 1989). Type II amplification is described by the ADS being identical to the AUD as found with instability in *S. lividans* (Altenbucher and Cullum, 1984).

Genetic instability can lead to problems for industrial fermentations if sporulation (strain preservation/inoculation) and/or antibiotic production are lost. Secondary metabolism pathways are often affected as these tend to be located in distal regions out with the conserved core. In these regions of the *S. rimosus* genome inverted repeats (550kb) were found that are prone to amplification and deletion; the longest to be reported so far (Pandza *et al*., 1997). The *otc* cluster was located around 600 kb

from one of the chromosome ends and is therefore prone to deletion (**Fig 1.3**, Pandza *et al*., 1997). PFGE analysis of the R6 strain showed that amplification frequently occurred, especially when spores were used for inoculation. Mutants with a wide range of varying phenotypes were produced (sporulation levels, pigment production, colony morphology, levels of OTC production/resistance) that were split into three phenotypic classes (Gravius *et al*., 1993).

Class I mutants were 99% of isolated variants and could not sporulate well. They encoded resistance but could not synthesise OTC. Class II mutants were ~1% of variants and were OTC-sensitive. They contained a large deletion, around 750kb that included the *otc* cluster and were phenotypically uniform. PFGE analysis showed that one end of the chromosome had been deleted. Class III mutants were 0.1% of isolated variants and displayed increased resistance to OTC. They had increased levels of OTC production coinciding with increased pigmentation. Most Class III variants showed a reproducible large-scale production of OTC, which most likely included a deletion and a low-level reiteration of a DNA rearrangement. It was found that a 200kb fragment containing the *otc* cluster had been amplified by 3-4 times and the distal chromosomal sequences had been deleted These mutants were phenotypically uniform but were often unstable and unable to maintain elevated OTC production, thus were not stable enough for industrial fermentations (Gravius *et al*., 1993). For further information on the genetics of each class, see Gravius *et al*., (1993), Hranueli *et al*., (1999) and Denapaite *et al*., (2005). For a detailed review on *S. rimosus* genetics, see Petkovic *et al*., (2006).

One of the main goals for investigating the molecular genetics of the *otc* cluster is to develop novel approaches by creating hybrid PKS systems using 'combinatorial biosynthesis' (Kantola *et al*., 2003; Hranueli *et al*., 2005; Hopwood, 1997). It would be ideal to be able to synthesise polyketides of potential therapeutic value *in vivo* that would otherwise be difficult to synthesise via conventional techniques. Data generated from characterized mutants of specific genes have led to prototype models of these novel PKS systems based on the blueprint of tetracycline (McDaniel *et al*., 1995; Petković *et al*., 1999; Khosla and Tang, 2005; Perić-Concha *et al*., 2005).

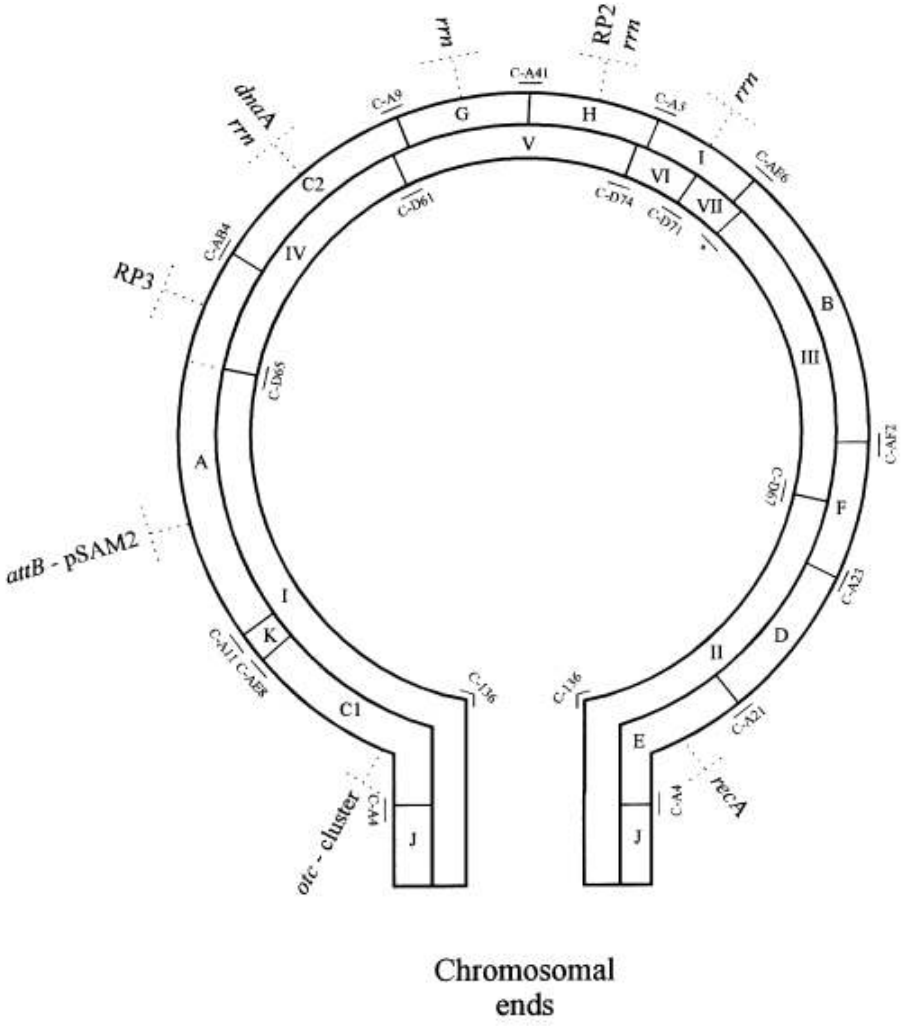


Fig 1.3 Genomic linkage map of *S. rimosus* R6

The *otc* cluster lies approximately 600kb from the end of the linear chromosome. Taken from Pandza *et al*., (1997).

1.4 Polyketides and secondary metabolites

Secondary metabolites are compounds produced at a late stage of growth that can aid but may not be necessary for the survival of the organism (Vining, 1992). They can be as diverse in structure as in function and are often biologically-active. Micro-organisms produce secondary metabolites during the stationary phase of growth in liquid media or during differentiation on solid media (Chater and Merrick, 1979). Secondary metabolites are also produced by eukaryotes such as fungi, lichens and plants. Their actions usually fall into one of two mechanisms: they can act within the host organism to assist the development of the cell e.g. primary metabolism (Vanek *et al*., 1981) or secreted by the host to act on neighbouring foreign bacteria that may be of threat or competition.

Polyketide antibiotics are classic examples of secreted secondary metabolites amongst others such as mycotoxins, insecticides and herbicides. They have been researched ever since John Norman Collie pioneered the earliest studies of the compounds in the early 1900's (Collie, 1907). As technology for molecular biology has advanced, so has the rate of biochemical characterization of the genetic pathways. Therapeutic polyketides have been of long term commercial value to the pharmaceutical industry. Examples include; antibiotics (tetracyclines and erythromycin), immunosuppressants (rapamycin, Vezina *et al*., 1975), anti-cancer compounds (anthracyclines - doxorubicin), polyene anti-fungal agents (amphotericin (Oura *et al*., 1955), rimocidin (Davisson *et al*., 1951), veterinary products (monensin, Haney and Hoehn, 1962), agricultural products (avermectin, Burg *et al*., 1979) and cholesterol-lowering agents (lovastatin, Endo *et al*., 1976). Polyketides are produced by complex and highly regulated pathways and often unique to the species that make them. This leads to the conclusion that secondary metabolites must have been important to the evolutionary survival of bacteria. It is unlikely that they evolved from primary metabolism, as there would be no need for such highly-regulated pathways if they were not a necessity for the survival of the organism (Maplestone *et al*., 1992).

Polyketides exist in many different structural forms, but most fall into one of two classes: aromatic or complex. Aromatic polyketides are formed by the iterative

decarboxylative aldol condensations of metabolic building units such as acetyl- or malonyl-CoA and the subsequent catalysed 'folding' of this polyketide chain (Magnuson *et al*., 1993). The mainly unreduced polyketone backbone (-CH_2-CO-)n is aromatised/cyclised to give upwards of 2 aromatic rings. Side chains and/or functional groups vary between compounds and can have a profound influence on pharmaceutical properties. Aromatic polyketides are synthesised by a diverse series of catalysed reactions and the unique combinations of catalysed modifications encoded by each specific pathway allows huge diversity. Complex polyketides, e.g. erythromycin A (Cortes *et al*., 1990), are synthesised from mainly reduced polyketide chains. The reduced backbone prevents aldol condensation reactions and therefore, the formation of aromatic rings (Donadio *et al*., 1991). The resulting long acyl chains are cyclised and diversified through a much more complicated system than those of the aromatic polyketides.

The biosynthesis of polyketides by polyketide synthases (PKS) is analogous to fatty acid biosynthesis (Hopwood and Sherman, 1990). The biosynthesis of fatty acids occurs via one of two mechanisms: Type I or Type II fatty acid synthase (FAS) complexes. The biosynthesis of most fatty acids/polyketides involves a group of closely regulated and sometimes co-transcribed genes that produce the so-called 'minimal FAS/PKS'. This is comprised of a condensing enzyme component, β-ketoacyl synthase (KS) composed of an α and β subunit and an acyl carrier protein (ACP) (Kim *et al*., 1994). Together, they control the iterative decarboxylative condensation of acyl-CoA extender units (often acetyl- or malonyl-CoA) after the priming of a specific acyl starter unit on to the thiol group of the free 4'-phoshopantetheine arm of the ACP (**Fig 1.4)**. See Hopwood and Sherman, (1990) for further details on fatty acid biosynthesis.

Polyketides vary in mode of function. For example, the tetracyclines and macrolides are bacteriostatic compounds that function by inhibiting protein synthesis of bacteria (Ohnuki *et al*., 1985). Others like the rifamycins and the anthracyclines interfere with RNA and DNA synthesis, respectively (Lewin, 1994). The biosynthesis of all polyketides is very similar. In *Streptomyces*, polyketide biosynthesis is normally triggered by environmental stimuli such as depleting carbon resources or tissue differentiation (Chater and Merrick, 1979). Three types of PKS have been described.

Type I PKS: analogous to Type I FAS clusters (Wakil, 1989). They function as large multi-domain polypeptide 'Megasynthases'. Each domain has a specific enzymatic activity and they are closely. The biosynthesis of Erythromycin A (**Section 1.9**) from *Saccharopolyspora erythrea* (Cortes *et al*., 1990) and avermectin from *Streptomyces avermitilis* (Yoon *et al*., 2004) are well known examples.

Type II PKS: characterized by clusters of individual genes and the iterative functions of the dissociated enzymes/proteins encoded accordingly. They are closely analogous to Type II FAS clusters (McCarthy and Hardie, 1984). The biosynthesis of tetracycline/chlortetracycline from *S. aureofaciens*, oxytetracycline from *S. rimosus*, actinorhodin from *S.coelicolor* and tetracenomycin from *S. glaucesens* (Hopwood and Sherman, 1990; Hopwood, 1997) are examples.

Type III PKS – These polyketide synthase complexes were previously believed to be exclusively produced as secondary metabolites of plants. Chalcone synthase was the prototypical example of a Type III PKS and studies have helped contribute most to our understanding of these polyketides (Kreuzaler and Hahlbrock, 1975; Austin and Noel, 2003). However, Type III PKS' have more recently been found in bacteria. (**Section 1.10,** see **Fig 1.5** for linear alignment of FAS/PKS genes/gene clusters).

1.5 Type II PKS biosynthesis: Actinorhodin

Early studies of *S. coelicolor* observed production of a pigment on complete medium that had different properties depending on pH levels. In acidic conditions, this pigment was insoluble, red and not bioactive but in alkaline conditions was soluble, blue and exhibited broad-spectrum antimicrobial activity (Wright and Hopwood, 1976a). Mutants that could not produce the pigment when exposed to ammonia fumes had no antimicrobial properties (Wright and Hopwood, 1976a). The compound responsible was the 16C benzoisochromanequinone (Wright and Hopwood, 1976a) acid-base indicator antibiotic, actinorhodin (ACT) (Wright and Hopwood, 1976b; Brockmann, 1966) (**Fig 1.7h**). The biosynthesis of this antibiotic is described here as the most significant polyketide produced by a Type II PKS in *S. coelicolor*. The elucidation of the biosynthetic pathway has been crucial towards the genetic and biochemical understanding of aromatic polyketide biosynthesis.

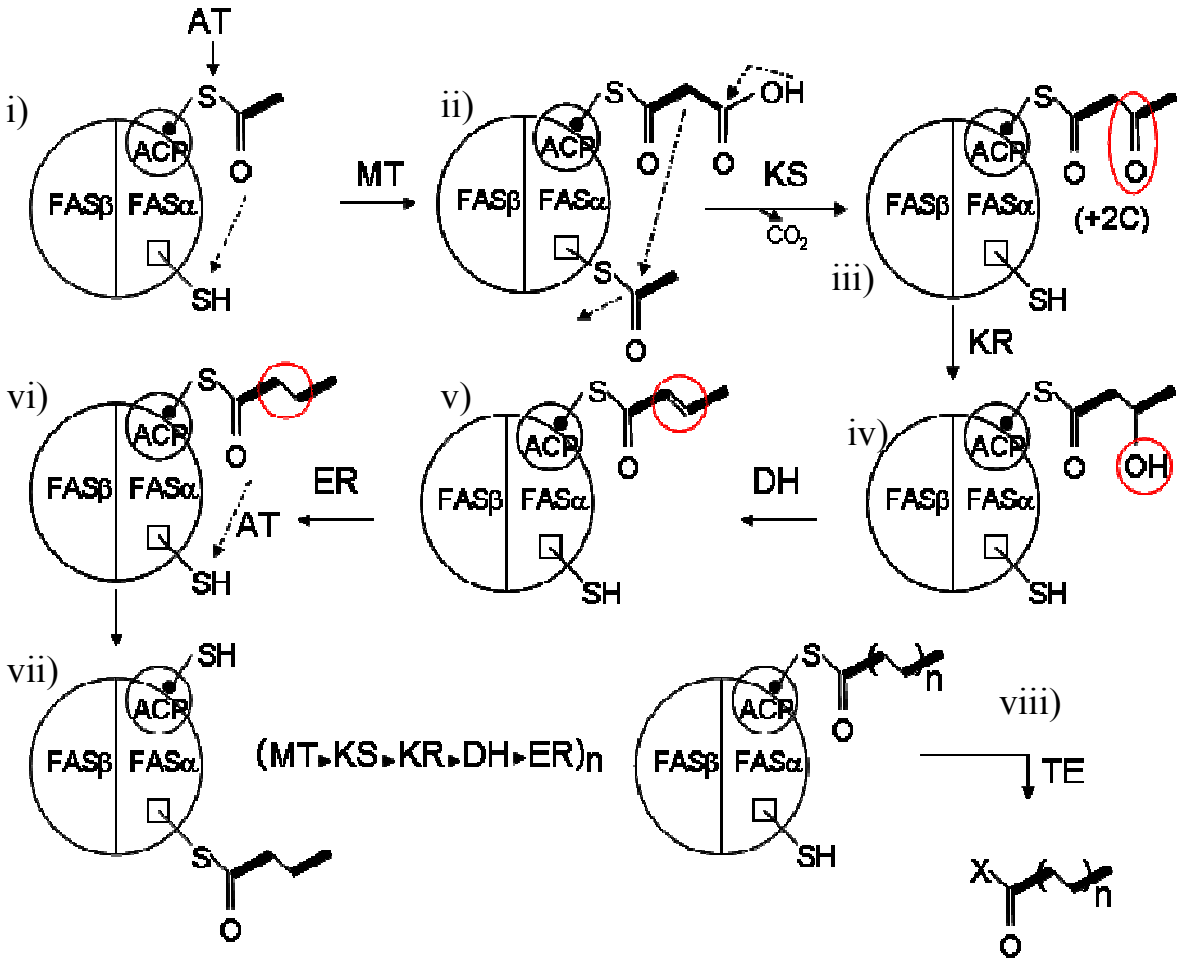


Fig 1.4 Fatty acid biosynthesis as an analogy to polyketide biosynthesis

i) The acyl thioester starter unit is transferred by an acyltransferase (AT) to the active site cysteine of the ketosynthase (□) (KS); ii) Malonyl transferase (MT) then transfers an extender unit (malonyl-CoA) to the free 4'-phoshopantetheine arm of the ACP (●); iii) the KS then catalyses condensation between the starter and extender unit; iv) A ketoreductase (KR) reduces this intermediate followed by v) dehydration by a dehydratase (DH) and vi) enoyl reduction by an enoyl reductase (ER); vii) the resulting butyryl-ACP intermediate is then transferred back to the active cysteine of the KS, completing a 2C extension of the initial starter unit; the extended unit acts as the primer for condensation of the next extender unit and the cycle repeats until the chain reaches its full length, at which point viii) it is cleaved by a thioesterase (TE) (Adapated from Hopwood and Khosla, 1992).

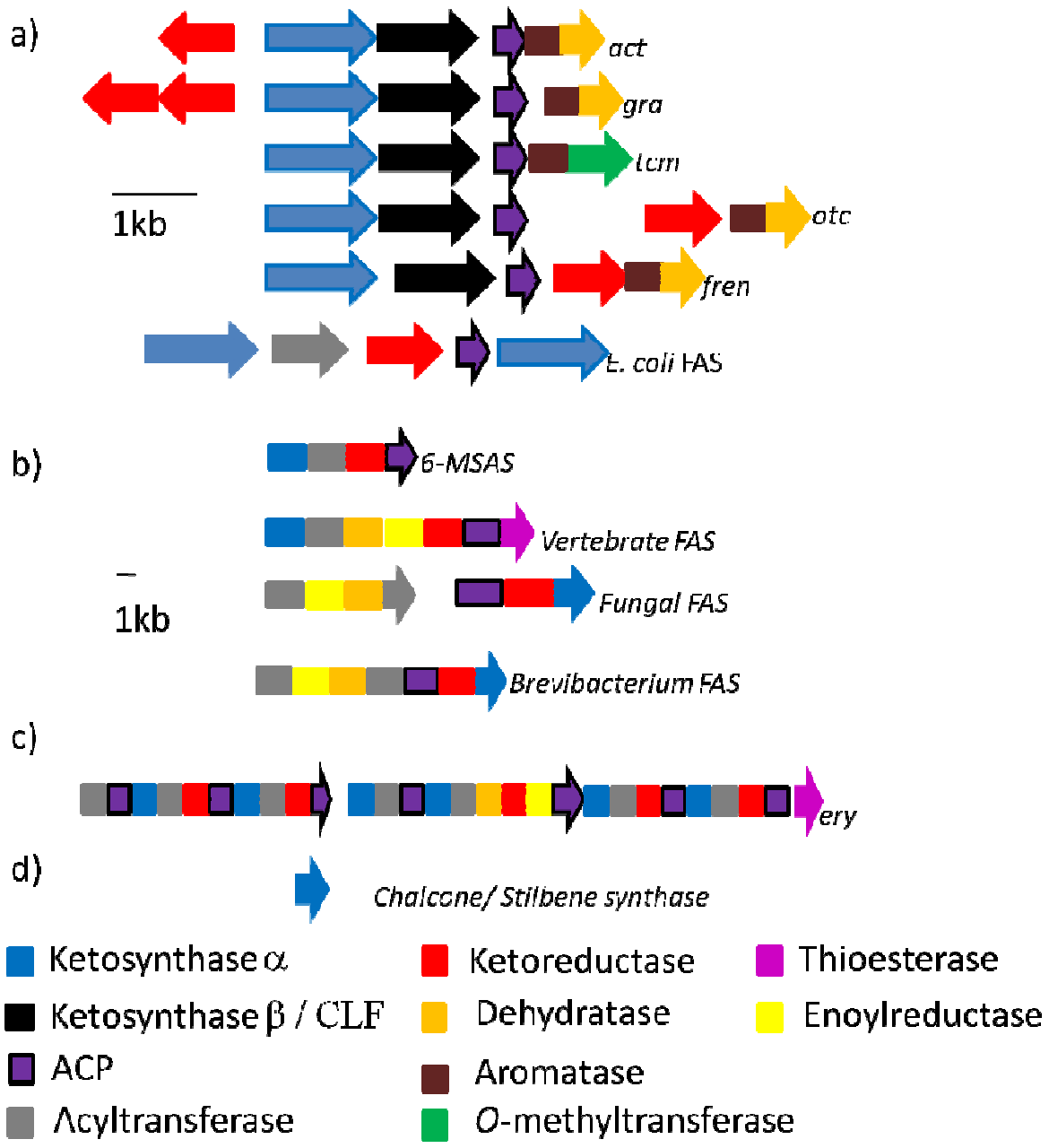


Fig 1.5 Basic linear alignment of selected FAS/PKS clusters

a) Type II PKS's (*act*, actinorhodin; *gra*, granaticin; *tcm*, tetracenomycin; *otc*, oxytetracycline and *fren*, frenolicin) (see **Section 1.5-1.8**)

b)/c) Type I PKS's/FAS's (*ery*, erythromycin and *6-MSAS*, 6-methylsalicylic acid) (see **Section 1.9**)

d) Type III PKS (see **Section 1.10**).

(Adapted from Hopwood and Khosla, 1992; Hopwood, 1997)

Mutants deficient in actinorhodin biosynthesis were characterized by co-synthesis tests (Rudd and Hopwood, 1979). Each mutant was classified into one of seven groups (*actI-actVII*) depending on their ability to synthesise actinorhodin by acting as the 'secretor' or 'convertor' to the other mutants. Only class II mutants were unable to act as either, indicating a role in regulation or polar effects caused by mutations. The mutants were subsequently classified in the following order; *actI*, *actIII*, *actVII*, *actIV*, *actVI*, *actV* (Rudd and Hopwood, 1979).

The biosynthetic genes as well as at least one regulatory gene in the *actII* locus were mapped by complementation (Rudd and Hopwood, 1979). The entire cluster was later located on a DNA fragment approximately 26kb in size (Malpartida and Hopwood, 1984; 1986). The gene for self-resistance to the antibiotic was also located within the cluster when the plasmid containing the whole cluster, pIJ2303, could not only complement any of the seven *act* mutants but also expressed actinorhodin biosynthesis and resistance when transformed into the actinorhodin-sensitive *S. parvulus* (Malpartida and Hopwood, 1984). The transcripts for the early genes (*actI, III*, *VII*, *IV*), responsible for synthesising the backbone of actinorhodin, were located on the right side of the cluster, while those responsible for more refined tailoring steps later in biosynthesis (*actVI*, *V*) were found mainly to the left. The transcripts for the putative regulatory genes were located between these two groups (Malpartida and Hopwood, 1986). The biosynthesis of actinorhodin is summarised by **Fig 1.7,** while the map of the cluster is illustrated in **Fig 1.6**.

The monomeric backbone of actinorhodin is derived from an acetyl-CoA starter unit followed by the decarboxylative condensation of 7 malonyl-CoA units to produce the hypothetical 16C octaketide (**Fig 1.7a**). This backbone is synthesised by the gene products of the *actI* locus. It contains three ORF's, co-transcribed with *actVII* and *actIV* involved in the intermediate stages of biosynthesis. *ActIII* is located adjacently to the *actI* locus but its promoter is transcribed divergently (Fernandez-Moreno *et al*., 1992). *ActI-ORF1* encodes a bifunctional enzyme that acts as a condensing enzyme (β ketoacyl synthase) with a conserved active site cysteine residue commonly found in similar FAS and PKS systems. ActI-ORF1 also has a serine residue in a Glycine-Histidine-Serine motif with putative acyltransferase function (Fernandez-Moreno *et*

al., 1992). The stop codon of *ORF1* and the start codon of *ORF2* overlap, ensuring an equimolar production of each protein.

The amino acid sequence of ActI-ORF2 has 49% similarity, 29% identity to ORF1 but lacks the catalytic residues of the latter. Therefore, it is likely that *actI-ORF1* and *ORF2* are transcribed and translated as a heterodimeric complex (Fernandez-Moreno *et al*., 1992). Homologous genes are found in other Type II PKS systems such as the tetracenomycin cluster of *S. glaucescens* (Bibb *et al*., 1989), the granaticin cluster in *S. violaceoruber* (Sherman *et al*., 1989) as well as the *otc* cluster in *S. rimosus* (McDowall *et al*., 1991). However, the *actI-ORF1* and *ORF2*-like genes are not translationally coupled (Bibb *et al*., 1994b) in the frenolicin cluster of *S. roseofulvus*. Various combinations of the minimal PKS genes from the actinorhodin, granaticin and tetracenomycin clusters were co-expressed with other early-intermediate genes (*actIII*, *actVII* and *actIV*) using a plasmid-based system (pRM5) in the ACT-sensitive *S. coelicolor* CH999 (McDaniel *et al*., 1993). Changing *ORF1* or *3* had no effect on the length of the novel polyketide produced. When using the *act ORF2*, a 16C novel polyketide, aloesaponarin II (discussed below) was produced, while a 20C compound, RM20, was produced when using the *tcm ORF2*. Thus, it was suggested that *actI-ORF2* and its homologues in other clusters function as chain length determining factors (CLF) (McDaniel *et al*., 1993).

ActI and *actIII* mutants were unable to co-synthesise actinorhodin together, indicating that they were both involved in the early stages of biosynthesis (Rudd and Hopwood, 1979). *ActIII* was hypothesised to encode a ketoreductase that reduced the ketone group at C-9 of the 16C octaketide to form a hydroxyl group, thus catalysing the formation of the first cyclised ring of ACT (Hallam *et al*., 1988, McDaniel *et al*., 1993). Whether this step occurs during or after the biosynthesis of the octaketide is unresolved. ActIII showed similarity to other known oxidoreductases (Hallam *et al*., 1988). A putative NADPH binding motif, Glycine-X-Glycine-X-X-Alanine was identified, suggesting NADPH as a co-factor (Hopwood and Sherman, 1990). *ActIII* was co-expressed with *actI* and *actVII* in *S. galilaeus* ATCC 31671, producer of the anthracycline polyketides, aclacinomycin and 2-hydroxyaklavinone. The 16C shunt product, aloesaponarin II, produced by *actVI* mutants, was synthesised.

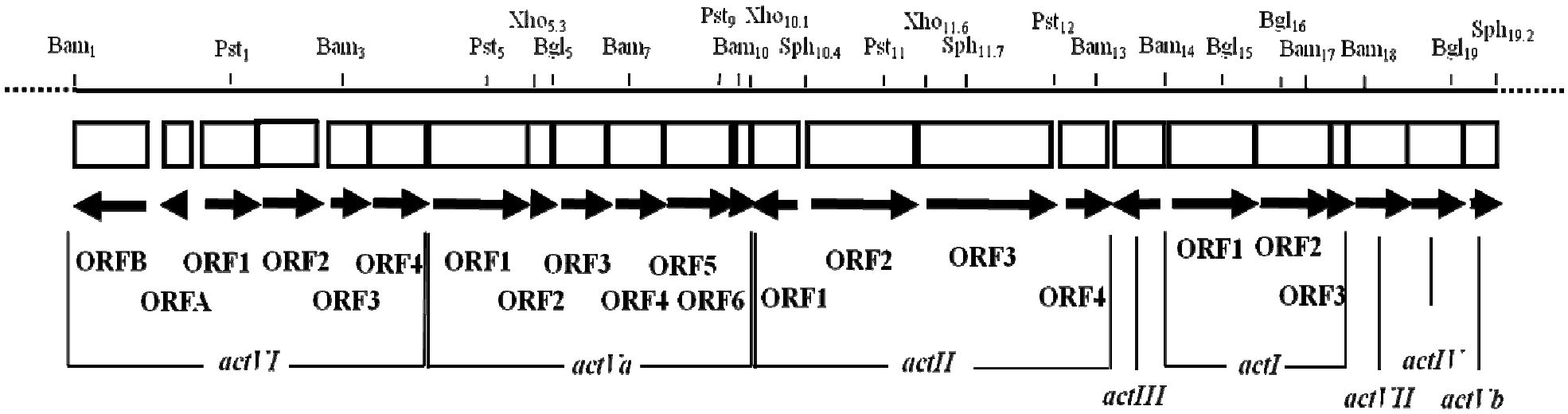


Fig 1.6 The *act* cluster

The (putative) products of each gene are described in the text. The arrows dictate the direction of transcription.

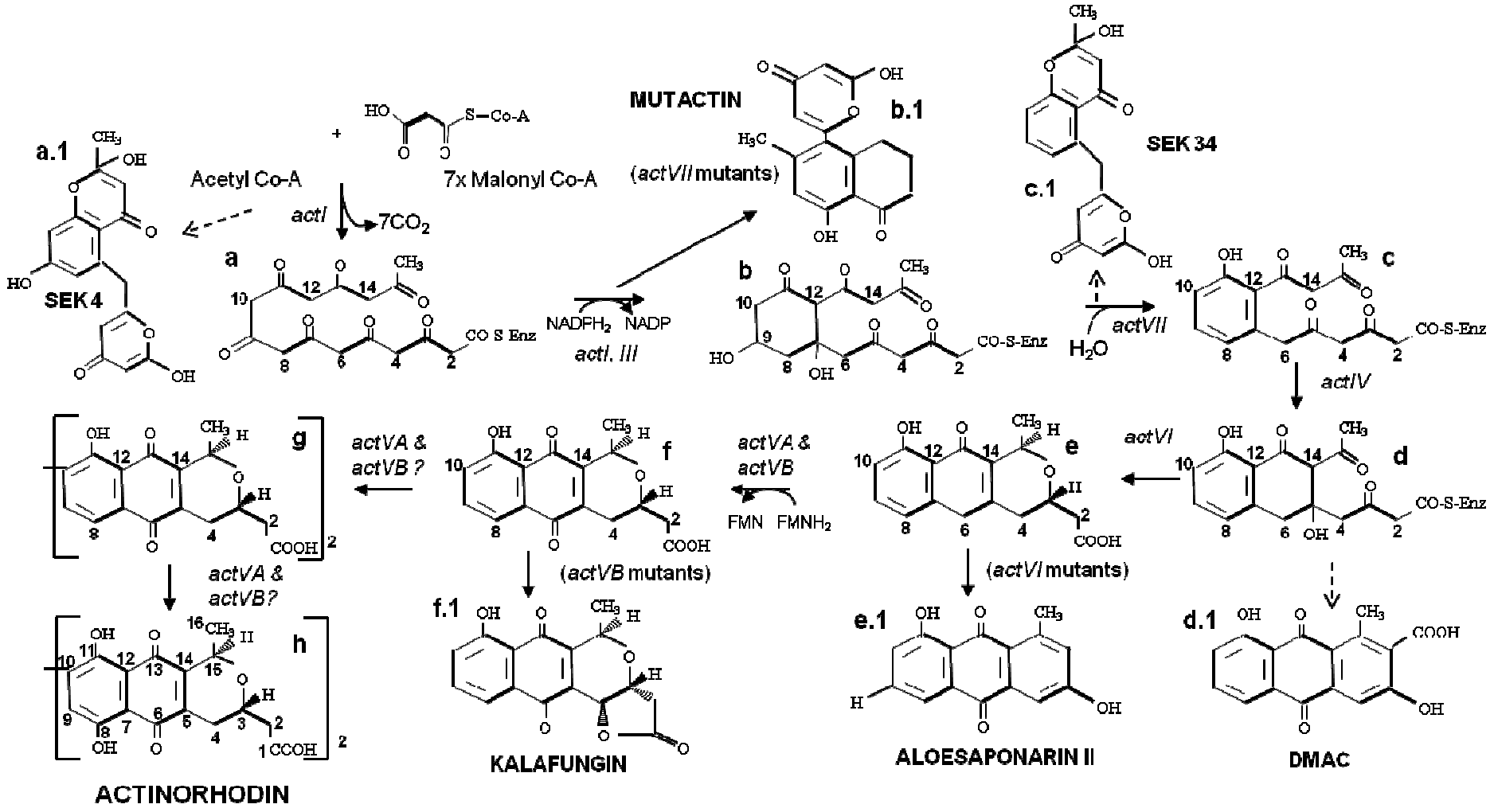


Fig 1.7 The biosynthesis of actinorhodin

See text for details. Dotted arrows indicate compounds isolated from co-expression of *act* genes in S. coelicolor CH999 (McDaniel *et al*., 1993)

Additionally, aklavinone was made by the recombinant strain instead of 2-hydroxyaklavinone, through reduction of the ketone group at C-9 (Bartel *et al*., 1990). When *actIII* was introduced independently, only aklavinone was produced, confirming that ActIII functioned as a ketoreductase at C-9 (**Fig 1.7b**) (Bartel *et al*., 1990). Analysis of RM20 indicated that it was also reduced at the C-9 ketone of the assembled polyketide chain (McDaniel *et al*., 1993).

Following reduction of the ketone at C-9. intramolecular aldol condensation between C-7 and C-12 forms the first benzoisochromanequinone ring, catalysed by the product of *actVII*. This is followed by a second aldolisation between C-5 and C-14 as well as dehydration of the C-9 hydroxyl group (Sherman *et al*., 1991). *ActVII* mutants acted as the earliest secretors during co-synthesis tests (Rudd and Hopwood, 1979). The *actVII* mutant, B40, synthesised the shunt product, mutactin (Zhang *et al*., 1990) (**Fig 1.7b.1**). The structure of this compound was produced due to aberrant intramolecular aldolisation resulting in a bond formation between C-6 and C-15 and a failure to dehydrate the C-9 hydroxyl group (Zhang *et al*., 1990). Successful complementation of the B40 mutant with *actVII* and *gra-ORF4* (from granaticin cluster) indicated that *actVII* encoded a bifunctional cyclase/dehydrase (Lydiate *et al*., 1985; Sherman *et al*., 1991).

The true functions of *actVII* and *actIV* were characterized from co-expression studies in *S. coelicolor* CH999 (McDaniel *et al*, 1993). Expression of the *act* minimal PKS produced the shunt product SEK4 (**Fig 1.7a.1**) and additional co-expression of *actIII* subsequently produced mutactin (Fu *et al*., 1994a). However, when *actI*, *actIII* and *actVII* were co-expressed, the shunt product SEK34/SEK34b was produced (McDaniel *et al*., 1994b). The structure of SEK34 (**Fig 1.7c.1**) proved that ActVII had no role in the formation of the second carbon ring of ACT but functioned as an aromatase that worked in conjunction with C-9 ketoreduction by ActIII to catalyse two dehydrations that form the first carbon ring (**Fig 1.7c**) (McDaniel *et al*., 1994b). Co-expression of *actI*, *actIII*, *actVII* and *actIV* produced the shunt product DMAC (**Fig 1.7d.1**) (McDaniel *et al*., 1993). Comparison of the structure of DMAC to SEK34 proved that ActIV acted as a cyclase that catalysed the aldol condensation between C-5/C-14 to form the second carbon ring (**Fig 1.7d**) (McDaniel *et al*., 1994b; Hopwood, 1997).

The formation of the heterocyclic actinorhodin precursor follows the activity of ActIV. The pyran ring is formed by reduction of the ketone group at C-3 and further dehydration and reduction to produce the heterocyclic structure (**Fig 1.7e**, Bartel *et al*., 1990). This reaction is blocked in *actVI* mutants, resulting in the synthesis of aloesaponarin II (**Fig 1.7e.1**, Bartel *et al*., 1990). A construct containing *actI*, *actIII*, *actVII* and *actIV* (pANT12) was introduced into the mutant strain, *S. galilaeus* 31133. [1,2-$^{13}C_2$] acetate was then incorporated into the synthesis of aloesaponarin II and analysis revealed that an aldol condensation between C-2 and the carbonyl group at C-15 followed by dehydrative carboxylation and spontaneous oxidation in air yielded aloesaponarin II (Bartel *et al*., 1990). Therefore, it was hypothesised that the pyran ring is formed by dehydration and further reduction at C-3 and C-15, respectively, by the products of the *actVI* locus (Fernandez-Moreno *et al*., 1994).

Analysis of a 5.7kb DNA fragment from which the original *actVI* mutants were mapped, revealed 6 ORF's in the order; *ORFB, ORFA, ORF1-4* (Malpartida and Hopwood, 1986; Fernandez-Moreno *et al*., 1994). Transcription of *ORF1-4* occurs 'rightwards' in the same direction as the *actVa* locus (**Fig 1.6**) and *ORFA* and *B* are transcribed 'leftwards'. Overlapping, divergent promoters were found in the intergenic region of *ORF1* and *ORFA*, of which at least one was controlled by the pathway activator protein encoded by *actII-ORF4* (**Section 1.8**). The stop codon of *ORF1* overlapped with the start codon of *ORF2* indicating translational-coupling. Likewise, the stop codon for *ORF4* overlapped with the start codon for *actVA-ORF1* (Fernandez-Moreno *et al*., 1994). The products of *actVI-ORF2* and *4* were very similar to each other, resembling the enoyl reductase domain of 6-DEB synthase (**Section 1.9**) as well as many alcohol dehydrogenases. Binding domains for the co-factors NADH and NADPH were also found. However, biochemically characterized roles for *ORF2* and *4* could not be assigned (Fernandez-Moreno *et al*., 1994).

The original *actVI* mutants were both assigned to *actVI-ORF1* (Malpartida and Hopwood, 1986). The amino acid sequence of ORF1 showed similarity to other known bacterial β hydroxybutyryl-CoA dehydrogenases (Fernandez-Moreno *et al*., 1994). Insertional inactivation studies of the *actVI* mutants with a series of phage vectors consisting of various fragments of the sequenced DNA showed that disruption of *actVI-ORFA, 1, 2* and *3* each led to significantly different phenotypes

and thus, were concluded to be directly involved with actinorhodin biosynthesis at different stages (Fernandez-Moreno *et al*., 1994). Disruption of *actVI-ORFB* and *ORF4* did not prevent actinorhodin biosynthesis, suggesting that *actVI-ORFA* signified the terminal gene of the *act* cluster (Fernandez-Moreno *et al*., 1994). Early bioinformatic data did not indicate a putative function for *ORFB* (Fernandez-Moreno *et al*., 1994). However more recent data have indicated a possible role as a β hydroxylacyl-CoA dehydrogenase (Hesketh and Chater, 2003).

Disruption of *actVI-ORFA* did not abolish ACT biosynthesis but lowered the yield, suggesting a potential regulatory role (Fernandez-Moreno *et al*., 1994). Updated BLAST analysis revealed a resemblance to known β hydroxylacyl-CoA dehydrogenases (Hesketh and Chater, 2003) although the isolation and characterization of an intermediate produced by an *ORFA* mutant, (*S*)-NHAB (1,4-naphthoquinone-8-hydroxy-3-[(3*S*)-acetoxy-butyric acid]), had led to the proposal that the gene product could be involved in stabilising the PKS complex (Taguchi *et al*., 2000; Ozawa *et al*., 2003). RT-PCR and Western blot analysis was later used to monitor the transcript levels of genes encoding the tailoring enzymes in an *actVI-ORFA* mutant in comparison to those of the wild-type. Transcription levels of *actVI-ORFA* and *ORF1* were reduced drastically and those of *actII-ORF1* and *2* from the *actII* locus, were also reduced (Taguchi *et al*., 2007). Transcript levels of *actI-ORF1*, *actII-ORF4*, *actIII* and *actVA-ORF3* were unchanged, which led to the hypothesis that the product of *actVI-ORFA* affects actinorhodin production by regulating genes involved in the later, tailoring steps of biosynthesis (Taguchi *et al*., 2007).

ActVI-ORF1 was ligated downstream of the *actIV* ORF under the control of the *actI* promoter into the plasmid pRM5 to form pIJ5660 and co-expressed in *S. coelicolor* CH999 (McDaniel *et al*., 1993; Ichinose *et al*., 1999). A yellow pigment was produced, and identified as the chiral actinorhodin precursor, (*S*)-DNPA (4-dihydro-9-hydroxy-l-methyl-10-oxo-3-H-naphtho-[2,3-c]-pyran-3-(S)-acetic acid) (**Fig 1.7e**) and a small amount of aloesaponarin II. It was thus concluded that ActVI-ORF1 functions as a stereo-specific reductase at C-3 (Ichinose *et al*., 1999). Homologues were subsequently identified; *gra-ORF6* from granaticin biosynthesis (Taguchi *et al*., 2001) and *med-ORF12* from medermycin biosynthesis (Li *et al*., 2005). *Gra-ORF6*

was also able complement a mutation in *actVI-ORF1* and act as the reductase on the C-3 keto group (Ichinose *et al*., 2001).

Disruption of *actVI-ORF3* led to the formation of a red precipitate that would later turn yellow and eventually blue, after 24-48hrs when exposed to ammonia fumes, indicating formation of actinorhodin via a slower, non-catalysed process (Fernandez-Moreno *et al*., 1994). This indicated that formation of the pyran ring could occur spontaneously via dehydration of the hypothesised hemiketal intermediate that would be produced after reduction of the C-3 ketone group by ActVI-ORF1 (Fernandez-Moreno *et al*., 1994). ORF3 had a putative function as a dehydratase/cyclase (Ichinose *et al*., 1999) and could assist the dehydration of the C-3 alcohol to form the cyclised pyran ring *in vivo,* that would otherwise occur spontaneously but inefficiently with a change in conditions such as a rise in pH (Ichinose *et al*., 1999).

It was not known whether ACT biosynthesis takes place while the bicyclic intermediate produced by ActIV is bound to an ACP intermediate in the PKS complex or acted on as an ACP-free substrate. The proposed bicyclic intermediate produced by ActIV was too unstable to synthesise; therefore, a closely-related and stable synthetic analogue, ONBA (3-Oxo-4-naphthylbutyric acid) and its corresponding *N*-acetylcysteamine thioester were synthesized. Recombinant ActVI bound and acted on the β keto group of the free acid form of ONBA instead of its thioester (Itoh *et al*., 2007), providing strong evidence that the tailoring steps take place with substrates in an unbound form (Itoh *et al*., 2007). Proteome mapping indicated that the products of *actVI-ORF2, 3* and *4* are all post-translationally modified and ActVI-ORF3 is believed to be exported to the cell wall (Hesketh and Chater, 2003), thus supporting the view that latter steps of ACT biosynthesis occur out with the PKS complex.

The original *actV* mutants produced an intermediate that could be converted to actinorhodin by all other mutants, except for those of the putative regulatory (*actII*) class. They were unable to convert any metabolite secreted by any other mutant, indicating that *actV* mutants were blocked in the final stages of actinorhodin biosynthesis (Rudd and Hopwood, 1979). Various mutants of the *actV* locus produced the shunt product kalafungin (**Fig 1.7f.1**) and an unstable yellow pigment

that could not be converted to kalafungin, which indicated that some mutants were blocked at a different stage in biosynthesis. Complementation of these mutants with phage vectors containing different fragments of the *actV* locus confirmed that the *actV* locus was split into two parts. Thus, some mutants were assigned to the *actVA* region and those that produced kalafungin to *actVB* (Rudd and Hopwood, 1979; Malpartida and Hopwood, 1986; Cole *et al*., 1987).

After the formation of the heterocyclic precursor, (*S*)-DNPA, further hydroxylation at C-6 and C-8 leads to the completion of actinorhodin biosynthesis. These hydroxylations are catalysed by products of the *actVA* locus, which consists of six ORF's, 1-6 (Caballero *et al*., 1991a). Hydroxylation at C-6 was shown to take place before C-8 because kalafungin was hydroxylated at C-6 but not at C-8 (Cole *et al*., 1987). It was also suggested that the presence of a lactone ring in the structure of kalafungin was caused by a C-4 oxidase, the gene for which may or may not be in the *act* cluster (Katz and Donadio, 1993). It was therefore proposed that the *actVB* mutant was not blocked at a hydroxylation step but was more likely to be responsible for the dimerisation of two molecules of a late-stage intermediate of ACT biosynthesis. This would then be recognised by the product(s) of the *actVA* locus responsible for hydroxylation at C-8 to complete the biosynthesis of actinorhodin (Fernandez-Moreno *et al*., 1992).

Most of the products of the *actVA* locus have yet to be characterized biochemically. The product of *ORF1* resembles the product of *actII-ORF2*, a putative transmembrane exporter protein involved in self-resistance to ACT (Caballero *et al*., 1991a; Caballero *et al*., 1991b; Fernandez-Moreno *et al*., 1991) while *ORF2, 3* and *4* were assigned as putative oxygenases (Caballero et al., 1991a). *ActVA-ORF6* was postulated to function as a monooxygenase that converts (*S*)-DNPA, to dihydrokalafungin (**Fig 1.7f**) using molecular oxygen to catalyse the reaction (Kendrew *et al*., 1997; 2000). This step is analogous to a similar step in tetracenomycin biosynthesis in which, the intermediate TcmF1 is converted to TcmD3 by the enzyme TcmH (Shen and Hutchinson, 1993), a reaction that ActVA-ORF6 was also able to catalyse (Kendrew *et al*., 1997).

Recombinant ActVB catalyzed the reduction of FMN by $NADH_2$ to $FMNH_2$ and NAD, thus confirming its role as a FMN:NADH oxidoreductase (Kendrew *et al*., 1995; Filisetti *et al*., 2003). A functional relationship between the product of *actVA-ORF5* and ActVB as a two-component flavin-dependent monooxygenase was proposed, believed to be involved in the final conversion of dihydrokalafungin (DHK) to dimerized actinorhodin (**Fig 1.7h**). Data showed ActVB to act as the FMN reductase and also indicated that the reduced FMN was thermodynamically transferred without interaction between the two proteins, to activate molecular oxygen, which consequently provides the energy for the monooxygenase activity of ActVA-ORF5 (Valton *et al*., 2004).

It was then illustrated that this reaction produced a FMN-hydroperoxide intermediate to act as the oxidant in the hydroxylation of DHK. It was postulated that the activity between the two enzymes would produce dimerized actinorhodin. Analysis illustrated that the quinone form of DHK was not accepted as a substrate by ActVA-ORF5, yet the corresponding hydroquinone was. However, despite confirmation of hydroxylated DHK, dimerized actinorhodin was not produced, thus the gene(s) responsible for this final step in actinorhodin biosynthesis remain unidentified (Valton *et al*., 2006).

1.6 The Tetracyclines

The tetracyclines, named accordingly due to their cyclic 4-ring aglycone core, are produced exclusively by *Streptomyces* using Type II PKS clusters. They were the first family of antibiotics found to work against both Gram-positive and Gram-negative bacteria, thus termed broad-spectrum antibiotics (Chopra *et al*., 1992). They have been widely used in human and veterinary medicine, which has led to widespread bacterial resistance, thus reducing their therapeutic efficacy (Neu, 1992; Levy *et al*., 1999). Tetracyclines have been used successfully in the treatment of various infections caused by e.g. mycoplasmas, chlamydiae and rickettsiae (Hacking, 1986; Chopra *et al*., 1992). Oxytetracycline in particular, has been heavily used in aquaculture over the last ten years, accounting for an overall increase in the annual global use of tetracyclines (Kerry *et al*., 1995; Alcaide *et al*., 2005). The most important producers of tetracyclines are *S. aureofaciens* and *S. rimosus* (Mitscher,

1978). The former produces a mixture of chlortetracycline (Duggar, 1948) and tetracycline, the latter a mixture of oxytetracycline and a small amount of tetracycline (Behal and Hunter, 1995).

Despite the prevalence of tetracycline resistance in clinical isolates, the compounds are still important in the treatment of some dangerous infections. Tetracycline is used as part of a successful 'triple therapy' combined with bismuth and metronidazole to treat stomach ulcers caused by *Helicobacter pylori* (Graham *et al*., 2005) and can also be used to combat some strains of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) (Lu and Holtom, 2005). The tetracycline derivative, doxycycline is used to treat malaria (Newton *et al*., 2005), reckettsial tickborne diseases (Suputtamongkol *et al*., 2004) and Lyme disease in its early stages before it reaches the central nervous system (Gunther and Haglund, 2005). Should an outbreak of anthrax ever occur, doxycycline could also be one of the drugs of choice for treatment (M'ikanatha *et al*., 2005).

Tetracyclines are also used for treatment of non-infective diseases by acting as inhibitors of angiogenesis and metalloproteinases (Lee *et al*., 2004; 2006). Non-antimicrobial derivatives are functional, indicating that the mode of inhibition is not due to antibiotic activity (Islam *et al*., 2003). They are also used as inducers of eukaryotic expression systems for many applications, including gene therapy (Goverdhana *et al*., 2005). Based on the mechanism of bacterial tetracycline resistance from transposon Tn*10*, the system uses a modified bacterial *tet* repressor that is fused to the activating domain of virion protein 16 of herpes simplex virus. This generates a tetracycline-controlled transactivator that can be expressed constitutively in HeLa cells (Gossen and Bujard, 1992).

Bacterial resistance to tetracycline is transmitted in transposon e.g. Tn*10* or plasmid form (Levy, 1989; Levy *et al*., 1999; Butaye *et al*., 2003) and has become one of the most commonly found examples of horizontal gene transfer of an antibiotic resistance determinant (Levy *et al*., 1999). Three mechanisms of resistance to tetracyclines are known; (i) energy-dependent cellular expulsion via efflux proteins, (ii) ribosomal protection and (iii) chemical inactivation (Speer *et al*., 1992). Around forty resistance determinants for tetracycline are now known (Roberts, 2005). Most

of the different tetracycline efflux proteins have been reported in Gram-negative bacteria: Tet proteins A–E, G, H, I, J, Z and Tet(30) are found exclusively in Gram-negative bacteria, whereas only TetK and TetL have been found mainly in Gram-positive organisms (Butaye *et al*., 2003; Roberts, 2005). Ribosomal resistance occurs more often in Gram-positive bacteria e.g. Tn*916*, which encodes TetM-mediated ribosomal resistance (Chopra *et al*., 1992). All tetracyclines work in a similar manner to inhibit protein synthesis (Ohnuki *et al*., 1985) by reversibly binding to the 30S subunit of bacterial ribosomes (Chopra, 1985), preventing the formation of the amino-acyl tRNA-ribosome complex, thus arresting cell growth.

The *otc* cluster in *S. rimosus* encodes two characterized resistance genes, *otrA* and *otrB*. A third, as yet uncharacterized gene, *otrC*, lies outside the cluster (Hunter, personal comm.) (**Fig 1.9**). OtrA was shown to protect the host by non-covalently modifying the ribosome, thus preventing OTC from binding the ribosome (Ohnuki *et al*., 1985; Doyle *et al*., 1991). OtrA has homology to TetM and TetO (Doyle *et al*., 1991). TetM and TetO are similar to elongation factors and function by releasing tetracyclines from the site of inhibition on the 30S subunit of the ribosome (Connell *et al*., 2003). Peptide sequence homology to the GTP-binding motif of two *E. coli* translational elongation factors, EF-G and EF-Tu, was also found (Zengel *et al*., 1984; Dittrich and Schrempf, 1992). However, further studies on the properties of OtrA as an elongation factor have yet to be published. Orthologues of *otrA* have been found in many other *Streptomyces* e.g. in *S. lividans* (Dittrich and Schrempf, 1992).

OtrB encodes an integral membrane protein that exports OTC from the mycelium (Ohnuki *et al*., 1985; Reynes *et al*., 1988; McMurry and Levy, 1998). The peptide sequence for OtrB is similar to the products of other efflux proteins such as the TetA(B) exporter encoded by Tn*10*. *S. rimosus* displays a resistance to OTC of at least 200μg.ml^{-1}. In mutants without functional copies of *otrA* or *otrB*, resistance drops dramatically but a low level is still detected, around 5μg.ml^{-1} (Binnie *et al*., 1989). It is therefore likely that *otrC* is responsible for this low level of resistance (Hunter, personal comm.).

The tetracycline family have heavily substituted aglycone core structures and these are summarised in **Fig 1.8**. These functional groups have been shown to be essential

for the ability of the compound to bind the target 16S RNA of the 30S subunit of bacterial ribosomes (Levy, 1984; Brodersen *et al*., 2000). Critical functional groups for bioactivity include the keto-enol group at C-11, the C-12a hydroxyl group, the dimethylamine group at C-4 and the C-2 primary amide on the carboxamido group (R5). On the contrary, some of the opposite peripheral oxygen atoms have very little role in bioactivity (Brodersen *et al*., 2000; Pioletti *et al*., 2001; Zhang *et al*., 2008). The semi-synthetic 6-deoxytetracyclines such as doxy- and minocycline are more potent (Charest *et al*., 2005) since their more lipophilic properties allow them to pass through the cell membrane with greater efficiency than efflux resistance proteins expel the compound (McMurry *et al*., 1982). Therefore, understanding of the effects of oxygenation of the tetracyclines is a clear point of interest in the development of novel therapeutic tetracycline derivatives (Pickens and Tang, 2009).

Obtaining a full understanding of the genetic and biochemical pathways of the tetracyclines will facilitate the development of novel antimicrobial polyketides derived from the tetracyclines to combat bacterial resistance. One of the most significant breakthroughs has been the development of novel glycyl-glycyl-9-substituted derivatives of tetracycline (Sum *et al*., 1994). These compounds have been referred to as the 'glycylcyclines'. Development of these compounds through clinical trials has led to the FDA approval of Tigecycline (Wenzel *et al*., 2005).

Tigecycline has shown activity against many pathogens that have developed resistance to tetracyclines. These include methicillin-resistant *Staphylococcus aureus* (MRSA - resistance to multiple antibiotics is often observed, including vancomycin-insensitive strains), *Streptococcus pneumoniae, Enterococcus* spp. (also including vancomycin-resistant strains) and some β-lactamase producing strains of *Enterobacteriaceae* (Felmingham, 2005). Another similar compound of this new class of tetracyclines currently in phase II clinical trials with broad spectrum activity against multi-drug resistant bacteria is PTK-0796, which has been developed by Paratek Pharmaceuticals (Butler, 2008).

1.7 The *otc* cluster and the biosynthesis of Oxytetracycline

A pathway to describe a general outline for the biosynthesis of tetracyclines was first presented by McCormick and Jensen from the Lederle Laboratories in the 1960's. This was based on mutant-based work on the pathway for tetracycline/chlortetracycline in *S. aureofaciens* (McCormick *et al*., 1962, 1963, 1965, 1968; McCormick, 1967; 1969). Mutants blocked at different stages of biosynthesis were used to isolate and purify stable intermediates, which provided the data to elucidate the pathway.

It was not until the work by Rhodes *et al*., (1981) at Pfizer that a linked biochemical and genetic study on the *otc* cluster in *S. rimosus* M4018 was published. The data suggested that there were two clusters on the chromosome. One was mapped to the '4 o'clock' position on the chromosome containing the gene loci *otcD, otcX, otcY* and *otcZ*, believed to be involved in the early stages of OTC biosynthesis. The second was located at '10 o'clock', containing the *otcA, otcB, otcC* and CSF1 genes, thought to be responsible for the later modifications of the polyketide.

A series of mutants were created by exposing spores to UV irradiation or the mutagen, NTG (Delic *et al*., 1970) that were incapable of producing OTC (Rhodes *et al.* 1981). These mutants were then characterized using co-synthesis tests, similar to those used in early studies of the actinorhodin cluster of *S. coelicolor* (Rudd and Hopwood, 1979, **Section 1.5**). Mutant pairs able to co-synthesise OTC were identified as being associated with the biosynthetic genes of the pathway. The tests produced 9 mutants that were each blocked at a specific point of OTC biosynthesis and were allocated into two classes; three mutants (class 1) were all blocked at the final stage of OTC biosynthesis, whereas six mutants (class 2) were blocked at six earlier intermediate stages. For further details, refer to Rhodes *et al*., (1981).

Rhodes *et al*., (1984) first cloned the *otc* resistance genes by constructing a gene bank in a high copy number vector and transforming the construct into an OTC-sensitive *S. rimosus* mutant, then selecting the resistant recombinants. This approach led to the identification of 2 resistance genes, *otrA* and *otrB*, each conferring resistance to OTC. The same two genes were also isolated from tetracycline-resistant

colonies of the OTC-sensitive *S. griseus* after DNA from the ATCC 10970 strain was cloned and transformed into the former (Ohnuki *et al*., 1985). Pfizer cloned the biosynthetic genes of the *otc* cluster using 'chromosome walking' (Hopwood *et al*., 1985) on the hypothesis that the biosynthetic and resistance genes must be closely-linked and found the resistance genes to be approximately 30kb apart illustrated by Southern blot analysis of a cosmid library containing the *otc* genes (Butler *et al*., 1989). The presence of the biosynthetic genes between the resistance genes was also confirmed after mutants blocked in the early stages of OTC biosynthesis (Rhodes *et al*., 1981) successfully synthesised OTC after sections of DNA between the resistance genes were cloned into the mutants (Butler *et al*., 1989).

Binnie *et al*., (1989) cloned the *otcC* gene. The product of this gene catalyses the hydroxylation of ATC to 5a,11a-(DHOTC) (Peric-Concha *et al*., 2005), one of the later steps in OTC biosynthesis (**Fig 1.10k-l**). The enzyme was purified and the N-terminal peptide sequence was used to synthesise complimentary oligonucleotides (Butler and Gedge, 1989). These were then used as probes for hybridization to the *otc* cluster. The data indicated that the gene was located amongst the 'early' genes rather than the 'late' genes, as suggested by Rhodes *et al*., (1981). This led to the proposal that the *otc* cluster existed as a single cluster flanked by the two resistance genes. This was proven when the entire section of DNA including the resistance genes was cloned in a 34kb *Eco*RI fragment into a low copy vector and transformed into the OTC non-producing strains, *S. albus* and *S. lividans*. OTC was successfully synthesised, thus proving that the *otc* cluster existed as a single unit (Binnie *et al*., 1989). Southern blot and restriction analysis of the *otc* cluster in *S. rimosus* R6 later revealed the same genetic architecture (Hranueli *et al*., 1999). The entire *otc* cluster was cloned and sequenced from *S. rimosus* 15883 and revealed 21 ORF's (Hunter and Hill, 1997) and more recently, the cluster from the ATCC 10970 (R7) strain was also sequenced (Zhang *et al*., 2006). The *otc* cluster is illustrated in its entirety in **Fig 1.9** and the pathway for OTC biosynthesis is shown in **Fig 1.10**.

The significance of having the resistance and biosynthetic genes in close proximity made teleological sense; the transfer of the cluster arranged as a single unit to a different species would aid the survival of the recipient, be it a new generation of the host species or a completely different species (Stone and Williams, 1992). This

genetic architecture was also found in the clusters for actinorhodin and tetracenomycin (Malpartida and Hopwood, 1984; Motamedi and Hutchinson, 1987). In some cases, the resistance gene is located amongst the biosynthetic genes, possibly to ensure that resistance is always expressed to coincide with antibiotic biosynthesis. It may also have a regulatory effect on the rate of antibiotic production (Chater and Hopwood, 1989). This is found in the clusters for methylenomycin in *S. coelicolor* (Chater and Bruton, 1985), erythromycin A in *Saccharopolyspora erythraea* (Bibb *et al*., 1986) and also for the aminoglycosides, neomycin in *S. fradiae* (Janssen *et al*., 1989) and hydroxystreptomycin, in *S. glausescens* (Vögtli and Hutter, 1987).

Two promoters for the *otrA* gene were identified; *otrAp1* and *otcCp1* (McDowall *et al*., 1999). *OtrA* is transcribed from *otrAp1* as a monocistronic unit during the exponential growth phase. At this point OTC production is repressed, therefore *otcCp1* is silent. Growing cells would need to be resistant to OTC produced by mature, neighbouring cells, thus explaining why two mechanisms for the transcription of *otrA* are present. OTC biosynthesis is induced at stationary phase, therefore *otrAp1* becomes silent and *otrA* is instead transcribed from *otcCp1* via read through from *otcZ* as the final gene as a polycistron including *otcC* and *otcZ*. Therefore, *otrA* is always transcribed, ensuring survival of the host (McDowall *et al*., 1999; Doyle *et al*., 1988; Doyle *et al*., 1991).

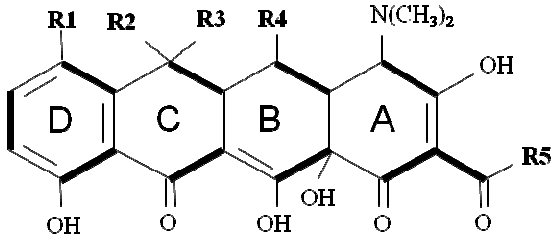
A putative repressor, *otrR*, was identified between *otrB* and *otcY1-1* (MacGregor-Pryde, 1995). The gene product is not similar to typical tetracycline repressors, but does have some similarity to some *E. coli* multi-drug repressors (Ramos *et al*., 2005). *OtrR* was found to be transcribed divergently to *otrB*, similar to that of Tn*10*, in which expression of the tetracycline efflux pump is also regulated by a divergent repressor. However, the functional relationship of *otrR* and *otrB* remains unclear.

The tetracyclines are unique due to the presence of a carboxamido functional group, believed to be derived from the incorporation of the unique starter unit, malonamyl-CoA (Thomas and Williams, 1983b). This unique characteristic has not been found in the biosynthesis of any other Type II polyketide. It therefore makes sense that a gene encoding a putative acyltransferase, *otcX3*, is present in the *otc* cluster.

Bioinformatic evidence supports the deduced role of *otcX3* as an acyltransferase. Type II PKS clusters that use standard starter units (acetyl-, malonyl-CoA, etc.) available from primary metabolism do not encode an acyltransferase. It was therefore hypothesised that the product of *otcX3* has a catalytic role in the binding and incorporation of malonamyl-CoA on to the thiol group on the free 4'-phosphopantetheine arm of the ACP/minimal PKS (Gatenbeck, 1961; Thomas and Williams, 1983a, 1983b) and presents the objective for this work. Orthologues of *otcX3* have been found in other gene clusters containing a gene encoding an acyltransferase e.g. *S. peucetius* (daunorubicin, Ye *et al*., 1994) and *S. galilaeus* (aklavinone and aclacinomycin, Chung *et al*., 2002) (**Chapter 3**), each of which incorporate propionyl-CoA, another uncommon acyl unit.

The hypothesis dictates that a mutant disrupted at *otcX3* would not produce OTC, but an analogue of OTC without the carboxamido group; 2-acetyl-2-decarboxamide-oxytetracycline (ADOT), which was previously isolated from an uncharacterized mutant of *S. rimosus* (Hochstein *et al*., 1960; Fu *et al*., 1994b) and has been shown to be a far less effective drug than OTC (Hunter, personal comm.). The 4C acetoacetyl-CoA would be used as a substitute starter unit from fatty acid metabolism (McCormick, 1967; Hochstein *et al*., 1960) (**Fig 1.8**). The presence of a unique carboxamido group in the chemical structure presents potential for chemical diversity from this group; therefore elucidation of the mechanism for the incorporation of malonamyl could lead to strategies for design of PKS gene manipulation approaches.

The presence of the gene, *otcY2-3*, in the cluster reinforces the hypothesis. Like *otcX3*, *otcY2-3* has yet to be biochemically characterized. OtcY2-3 has homology to a coumarate-CoA ligase used in the Type III PKS pathway for chalcone synthase (Schuz *et al*., 1983, see **Section 1.10**) that acylates the primer, coumarate - a similar situation as may occur in OTC biosynthesis with malonamate. If a standard starter unit such as malonyl- or acetyl-CoA were used, there would be no need for the presence of a CoA-ligase-like gene as these acyl units are readily available from primary metabolism. Thomas and Williams (1983a) used C^{13} to radiolabel malonate and acetate as precursors for OTC biosynthesis. From the NMR analysis of the products only the malonate unit was found to be incorporated into the formation of the carboxamido group indicating substrate specificity.



	R1	R2	R3	R4	R5
Tetracycline	H	OH	CH_3	H	NH_2
6-Demethyltetracycline	H	OH	H	H	NH_2
2-Acetyl-2-decarboxamide-tetracycline	H	OH	CH_3	H	CH_3
Oxytetracycline	H	OH	CH_3	OH	NH_2
2-Acetyl-2-decarboxamide-oxytetracycline (ADOT)	H	OH	CH_3	OH	CH_3
6-Deoxytetracycline	H	H	CH_3	H	NH_2
Chlortetracycline	Cl	OH	CH_3	H	NH_2
6-Demethylchlortetracycline	Cl	OH	H	H	NH_2
Minocycline	$N(CH_3)_2$	H	H	H	NH_2
Doxycycline	H	CH_3	-	OH	NH_2

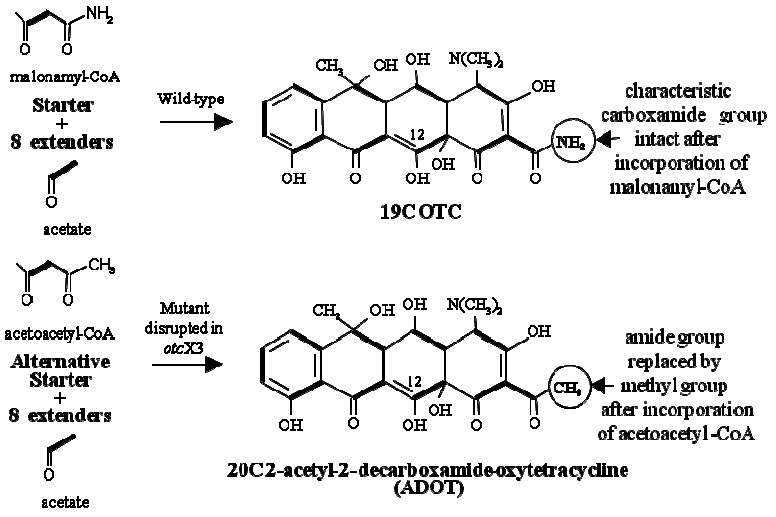


Fig 1.8 The tetracycline family and the hypothesized biosynthesis of ADOT

(Above) A summary of the chemical structures of the tetracycline family. (Below) The biosynthesis of oxytetracycline with carboxamido group intact in structure after incorporation of 3C malonamyl-CoA and the hypothesized incorporation of 4C acetoacetyl-CoA as an alternative starter unit in *S. rimosus* mutant disrupted in *otcX3* to produced the 20C ADOT (Hochstein *et al*., 1960).

Zhang *et al*., (2006; 2006b) conducted co-expression studies of *otc* biosynthetic genes based on the model provided by (McDaniel *et al*., 1993, **Section 1.5**). Data was published that was pertinent to the role of *otcX3*. The work described in this thesis was completed prior to the data published by Zhang's group. As such, these data are discussed in **Chapter 5**.

OTC biosynthesis commences via the transcription of the minimal PKS, analogous to the *actI* locus (ORF1-3) of the *act* cluster in *S. coelicolor* (Hopwood and Khosla, 1992, **Section 1.5**) and encoded by the products of the genes *otcY1-1*, *1-2* and *1-3* (Kim *et al*., 1994). *OtcY1-1* and *1-2* are translationally coupled. *OtcY1-1* encodes the ketoacyl:ACP synthase (KS) with an active site cysteine residue responsible for catalysing the iterative condensation of the nonaketamide backbone. *OtcY1-2* encodes the putative CLF (McDaniel *et al*., 1993; 1994a; Shen *et al*., 1995) and *otcY1-3* encodes the ACP. After the initial priming of the 3C malonamyl unit, the minimal PKS catalyses the iterative decarboxylative condensation eight 3C malonyl-CoA units to produce a 19C nonaketamide polyketide backbone (**Fig 1.10a**), which subsequently undergoes cyclisation/aromatisation (Kim *et al*., 1994).

Shunt products isolated from *in vivo* studies of co-expression of heterologous expression of minimum PKS genes from various clusters (actinorhodin, tetracenomycin, granaticin) indicated that the KSβ subunit was responsible for controlling the length of the polyketide (McDaniel *et al*., 1993). It is therefore likely that OtcY1-2 has a similar role in OTC biosynthesis. When the *otc* minimal PKS was co-expressed, only the malonate unit was found to be incorporated into the formation of the carboxamido group indicating substrate specificity (Kim *et al*., 1994).

In early studies of tetracycline biosynthesis, formation of the tetracyclic core was found to occur in a step-wise fashion. Mutants of *S. aureofaciens*, ED1369 (McCormick and Jensen, 1968) and S2242 (McCormick *et al*., 1968), produced the shunt products, protetrone and methylanthrone (**Fig 1.11m, n**), respectively. Analysis showed that the shunt products were produced as a result of the inability of the mutants to fully cyclise the nascent polyketamide. The structures also contained the carboxamido group, supporting the hypothesis that it was incorporated into the polyketide backbone at the start of biosynthesis.

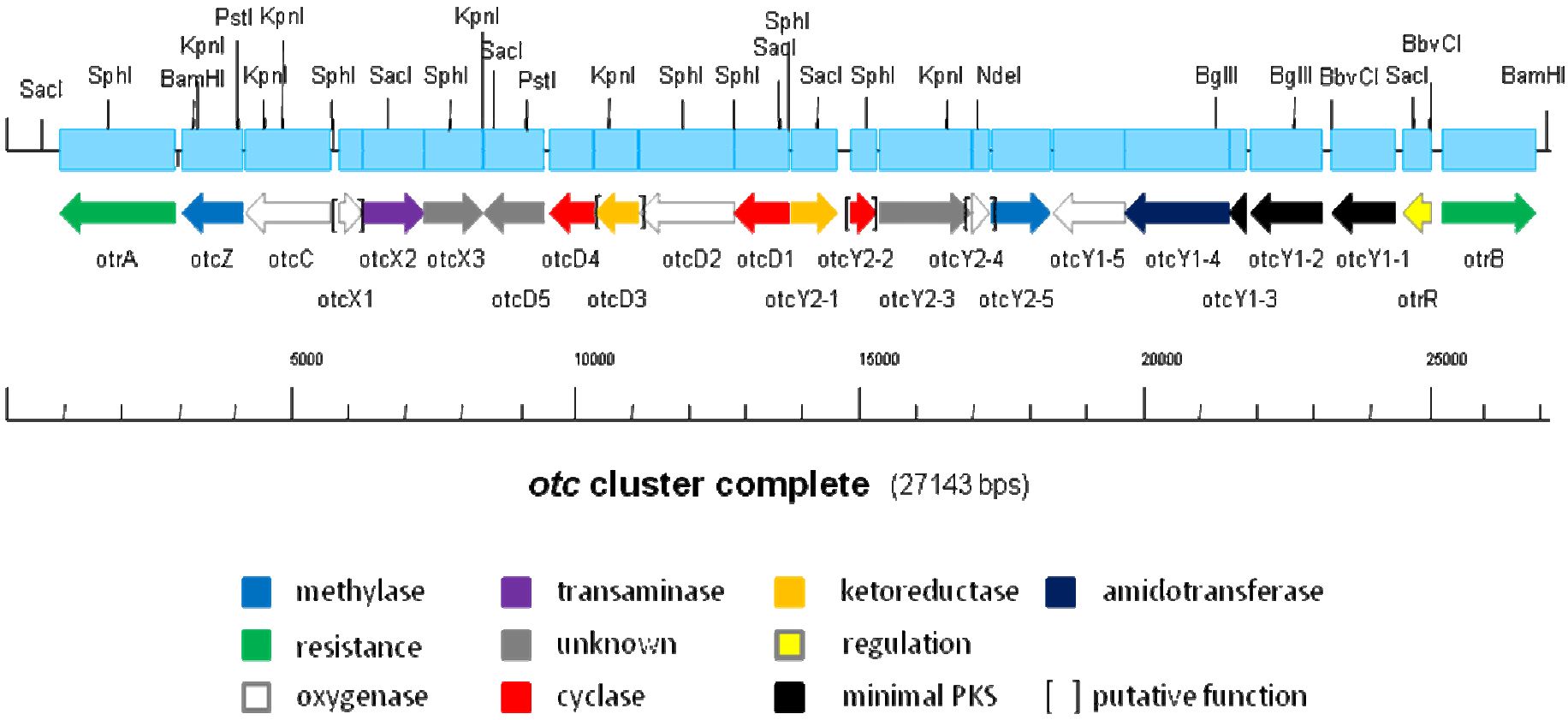


Fig 1.9 The *otc* cluster

The products of each gene are described in the text. The arrows dictate the direction of transcription

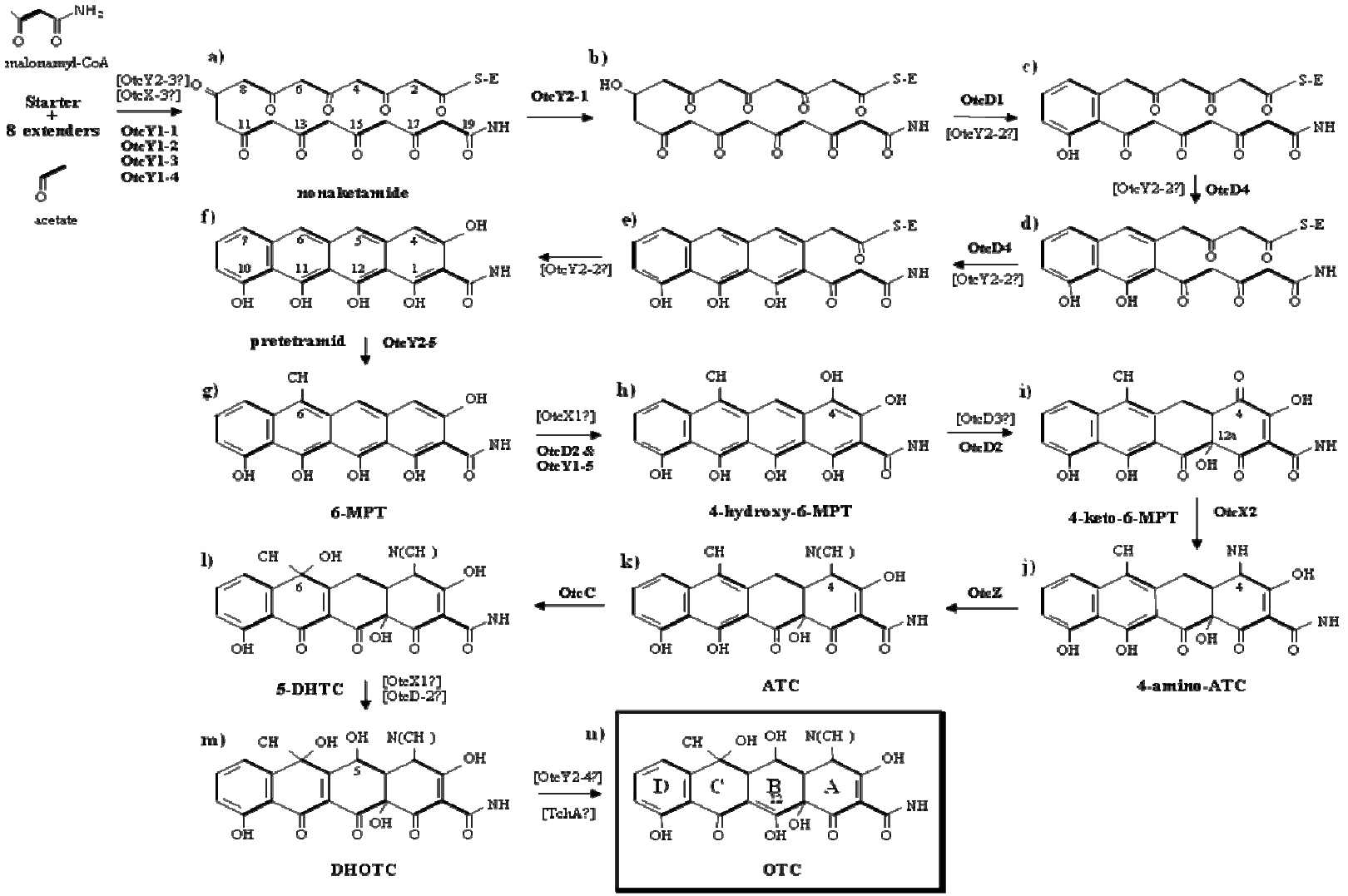


Fig 1.10 Flow chart illustrating OTC biosynthesis

Hypothesized elucidation of OTC biosynthesis, as of Wang *et al*., (2009). Adapted from Petkovic *et al*., (2006) [?] = putatively assigned role

Cyclisation of the first ring (Ring D, **Fig 1.10b-c**) involves an aldol condensation reaction, initially postulated to be catalysed by a C-9 ketoreductase (KR) encoded by *otcY2-1*, which is homologous to the *actIII* region of the actinorhodin cluster (Hopwood and Khosla, 1992; Fernandez-Moreno *et al*., 1992, **Section 1.5**). This was confirmed when novel alkaloids were produced from plasmid-based co-expression of various *otc* biosynthetic genes in *S. coelicolor* CH999. When *otcY2-1* was co-expressed, the compound produced confirmed that formation of a cyclised ring was the result of stereo-specific ketoreduction at C-9. In the absence of *otcY2-1*, a different compound was produced, the structure of which indicated spontaneous ring formation (McDaniel *et al*., 1993; Zhang *et al*., 2006; 2006b).

A second gene involved in the formation of ring D, is an aromatase/cyclase initially postulated to be encoded by *otcD1* (Hutchinson and Fujii, 1995). Upon disruption of *otcD1*, the mutant produced a series of novel truncated aromatic polyketides (LH1-4, **Fig 1.9a-d**), the structures of which proved the essential role of OtcD1 for the formation of the C-C aromatic bonds that form Ring D (Petkovic *et al*., 1999). Each structure also contained the carboxamido group, confirming the incorporation of malonamyl into the nascent polyketide (Petkovic *et al*., 1998; 1999). Aromatic rings were still formed in LH1-4, believed to be a result of spontaneous aromatisation following ketoreduction at C-9. *OtcY2-1* and *otcD1* could form a functional relationship as they are located adjacent to each other in the cluster and are involved in sequential steps of biosynthesis (Petkovic *et al*., 1998; 1999).

Zhang and co-workers later cloned *otcD1* and another putative cyclase, *otcD4,* into the plasmid pWJ35 (pRM5 derivative driving co-expression of *otcY1-1, 1-2, 1-3*, *1-4* and *otcY2-1*; McDaniel *et al*., 1993; Zhang *et al*., 2006; 2006b) to form pWJ83. When introduced into and expressed in *S. coelicolor* CH999, the novel compound WJ83T1 (**Fig 1.10f**) was isolated as the major product. The structure of WJ83T1 indicated that *otcD1* and *otcD4* were the only further essential genes required for the complete formation of rings A-D of OTC (Zhang *et al*., 2007).

Zhang *et al*., (2006) postulated that the putative cyclase encoded by *otcY2-2* was essential for cyclisation of the 4th ring of OTC but when co-expressed along with *otcD1* and *otcD4*, WJ83T1 (**Fig 1.11f**) was still synthesised. Therefore, OtcY2-2 was

concluded not to have an essential role in OTC biosynthesis. Additionally, co-expression of *dpsY*, a homologue of *otcD4* from daunorubicin biosynthesis in *Streptomyces C5* in place of *otcD4* in pWJ83 also produced WJ83T1, indicating relaxed substrate specificity (Zhang *et al*., 2007).

The earliest stable intermediate that was isolated in tetracycline biosynthesis was 6-methylpretetramid (6-MPT, **Fig 1.10f-g**, McCormick, 1967). Methylation at C-6 was proven to occur before cyclisation, as methylating and non-methylating mutants were able to synthesise tetraycline when fed 6-MPT (McCormick *et al*., 1963). It was proposed that methylation of pretetramid at C-6 was catalysed by the product of either *otcZ* or *otcY2-5*, assigned by Rhodes *et al*. (1981) as putative methylases. Later, *otcZ* and *otcY2-5* were individually cloned into pWJ83 to form pWJ120 and pWJ119, respectively (Zhang *et al*., 2007). Expression of pWJ120 in *S. coelicolor* CH999 still produced WJ83T1 (no C-6 methylation) as the major product. However expression of pWJ119 produced the compound, WJ119 (**Fig 1.11g**) which had methylation of the ketone group at C-6, thus proving that *otcY2-5* was responsible for methylating pretetramid to 6-MPT (Zhang *et al*., 2007). This made teleological sense since *otcY2-5* is located amongst the 'early' genes of the *otc* cluster (**Fig 1.9**).

6-MPT is then hydroxylated by an oxygenase to 4-hydroxy-6-MPT, first isolated from a mutant of *S. aureofaciens* (McCormick *et al*., 1965, **Fig 1.10g-h**). The *otcX* locus was initially assigned this function (Rhodes *et al*., 1981), but later sequence data revealed that *otcY1-5* or *otcD2* were more likely to be catalytic oxygenases (Zhang *et al*., 2006). *OtcX1* encodes a short polypeptide which has some similarity to the oxygenase encoded by *actVA-ORF2* from the actinorhodin biosynthetic cluster (McDowall *et al*., 1991; Caballero *et al*., 1991a). *OtcX1* may have a non-catalytic, co-operative role with either *otcY1-5* or *otcD2*.

4-hydroxy-6-MPT is reduced to the unstable 4-keto-anhydrotetracycline (4-keto-ATC) by a ketoreductase (**Fig 1.10h-i**, possibly involving the product of *otcD3*). This was then thought to be amidated by an aminotransferase at C-4 to form 4-amino-ATC (**Fig 1.10i-j**, McCormick *et al*., 1965). ATC is then formed by methylation of the 4-amino group to give a 4-methylamino group, followed by the subsequent methylation of that group and the reduction of the C=C double bond at C-4-4a. This

was deduced after an unstable intermediate, metatetrene (**Fig 1.11j**), was isolated from the *S. aureofaciens* mutant, T219 (McCormick *et al*., 1962). The mutant was deficient in the ability to reduce the double bond at C-4-4a, indicating that the amination and dimethylation steps occur prior to the reduction at C-4-4a.

Zhang and co-workers recently identified the essential *otc* genes for the formation of ATC. Four putative oxygenases; *otcY1-5*, *otcY2-4*, *otcD2* and *otcX1* along with the putative aminotransferase, *otcX2* and putative methylase, *otcZ,* were cloned into pWJ119 to create pWJATC1 (Zhang *et al*., 2008). ATC was isolated as the major product, when expressed in *S. coelicolor* CH999. ATC synthesis was abolished when *otcX2* or *otcZ* were removed, thus confirming their essential roles. Removal of *otcD2* from pWJATC1 also abolished ATC biosynthesis. However, WJ119 was still synthesised. ATC was still produced at the same yield upon removal of *otcY1-5*, *otcY2-4* or *otcX1*. Expression of the construct pWJATC4 (pWJ119 + *otcD2* + *otcX2* + *otcZ*) in *S. coelicolor* CH999 subsequently confirmed the biosynthesis of ATC (**Fig 1.10j-k**). Therefore, it was concluded that *otcD2* was the only essential oxygenase required to synthesise ATC (Zhang *et al*., 2008).

OtcD2 was cloned into pWJ119 and over-expression of the construct in *S. coelicolor* CH999 produced the compound WJ135 (**Fig 1.11i**). Recombinant OtcD2 was subsequently incubated with the OTC intermediate 6-MPT *in vitro*. HPLC/Mass spectrometry confirmed that 6-MPT had been converted to WJ135 by OtcD2. The structure of WJ135 indicated that 6-MPT had been hydroxylated at the C-4 position between rings B and A. In turn, this event would have created an analogue of the known unstable OTC intermediate, 4-keto-ATC, which would eventually break down between the B and the A ring, thus losing the A ring completely and leaving a truncated B ring structure that would result in the formation of WJ135. *OtcD2* was therefore biochemically characterized as an NADPH-dependent dioxygenase that hydroxylates 6-MPT at the C-4/12a positions (Zhang *et al*., 2008).

OtcD2 and *otcX2* were cloned into pWJ119 and HPLC analysis of the extracts from expression in *S. coelicolor* CH999 confirmed the biosynthesis of the known OTC intermediate, 4-amino-ATC (**Fig 1.10j**). Consequently, *otcX2* had been

biochemically characterized as a C4 reductive transaminase, the first of its kind found in any Type II PKS (Zhang *et al*., 2008).

OtcZ is located next to *otrA* (Butler *et al*., 1989). The OtcZ peptide sequence is similar to the C-terminus of TcmN of the tetracenomycin pathway in *S. glausescens*, which functions as an *O*-methyltransferase (Summers *et al*., 1992) and also showed homology to the peptide sequence of bovine hydroxyquindole *O*-methyltransferase (Ishida *et al*., 1987). The gene was then sequenced and characterized as a putative methyltransferase (McDowall, 1991; McDowall *et al*., 1999). Rhodes *et al*., (1981) proposed that *otcZ* was blocked before the conversion of 4-amino-ATC to ATC. Therefore, it was initially postulated that OtcZ was involved in the C-6 methylation of the nonaketamide chain before cyclisation/aromatisation into 6-MPT since it was the only known methylation step prior to the formation of 6-MPT (Butler and Gedge, 1989; McDowall, 1991; McDowall *et al*., 1991).

The inability of the *otcZ* mutant to convert either 6-MPT or 4-amino-ATC to OTC (Rhodes *et al*., 1981) indicated that there may have been more than one mutation in the mutant strain, raising doubts over the role of the gene. A homologue of *otcZ* - *ctc6*, was isolated from the *ctc* cluster of a strain of *S. aureofaciens* that produced 6-demethyl-chlortetracycline (Dairi *et al*., 1995) despite the presence of the methylase, *ctc6*. This suggested that the function of *otcZ* (Rhodes *et al*., 1981) had been mis-assigned. Alternatively, *ctc6* could contain a point mutation causing loss of function of the methylase. DNA screening of *otcZ* against the chlortetracycline cluster in *S. aureofaciens* indicated that *otcZ* had sequence similarity to *ctc-ORF2*, which was characterized as a C-4-*N*-methylase (Ryan *et al*., 1994; 1996).

Recombinant OtcZ was then assayed with the OTC intermediate 4-amino-ATC and *S*-adenosyl-methionine (SAM). Analysis of short-term incubation by HPLC revealed a single methylation of the C-4 amino group, with longer incubation confirming dimethylation to complete the formation of ATC (**Fig 1.10k**). The presence of SAM was essential for the completion of the reaction. *OtcZ* had thus been characterized to encode an SAM-dependent N, N-dimethyltransferase; the first of its kind known to use an aromatic polyketide aglycone as its substrate (Zhang *et al*., 2008).

After establishing the minimum 'essential' genes to synthesise ATC, Wang *et al*., (2009) investigated the role of a 'non-essential' gene in OTC biosynthesis, by studying the metabolites produced by a Δ*otcY1-5* mutant. Bioinformatic data had shown that *otcY1-5* has a homologue in the mithramycin cluster (Prado *et al*., 1999), which led to the assignment of *otcY1-5* as a putative FAD-dependent oxygenase. As expected, HPLC analysis using UV absorbance at 360nm of metabolic extracts of the Δ*otcY1-5* mutant confirmed that OTC biosynthesis had not been abolished. However, further analysis at 430nm and subsequent NMR analysis revealed a novel shunt product, PW1 (**Fig 1.11i**), which was not synthesised by the wild-type. It was also observed that the yield of OTC produced by the Δ*otcY1-5* mutant was ~50% lower than the wild-type. In the same work, a Δ*otcD2* mutant was created and concurrent HPLC/NMR analysis of the Δ*otcY1-5* mutant confirmed the loss of OTC biosynthesis by the Δ*otcD2* mutant but showed production of another shunt product, PW2 (**Fig 1.11j**) absorbing at 430nm, which was also synthesised by the wild-type but at a much lower concentration (Wang *et al*., 2009).

PW1 is likely synthesised by the Δ*otcY1-5* mutant as a result of inefficient hydroxylation of the intermediate 6-MPT at C-4 to form 4-hydroxy-6-MPT. Therefore, it is likely that OtcD2 catalyses this reaction at a lower efficiency in the absence of functional OtcY1-5. It was thus concluded that OtcY1-5 subsequently catalyses the hydroxylation at C-12a of 4-hydroxy-6-MPT to form 4-keto-ATC (**Fig 1.11h-i**). Analysis of PW1 and PW2 showed glucuronation of the tetracyclic structure at the C-5 and C-4 positions, respectively, possibly catalysed by a glucuronosyltransferase endogenous to *S. rimosus*. This would result in the formation of PW1 and PW2, when combined with air oxidation or the action of another oxygenase. PW1 was not detected in the Δ*otcD2* mutant, thus confirming that the presence of *otcY1-5* suppresses oxidation at C-5. The detection of PW2 at lower concentrations in the wild-type was presumed to be caused by incomplete conversion of 4-hydroxy-6-MPT to 4-keto-ATC (Wang *et al*., 2009).

Removal of *otcD2* from the plasmid pWJATC4 (Zhang *et al*., 2008) (as described above) confirmed the loss of ATC production from expression in *S. coelicolor* CH999 but also led to the detection of the shunt product JX11 (**Fig 1.11k**), produced from spontaneous oxidation of 4-hydroxy-6-MPT. The heterologous production of

JX11 confirmed the role of OtcY1-5 as an ancillary enzyme of OtcD2. The presence of both genes in the wild-type induces a co-operative function to tailor the OTC intermediates to minimise the production of shunt products (Wang *et al*., 2009). The lack of glycosylation observed in the structure of JX11 also showed that OTC intermediates can be endogenously glycosylated by *S. rimosus*, but does not seem to occur in *S. coelicolor* (Wang *et al*., 2009).

ATC is hydroxylated at C-6, catalysed by the oxygenase encoded by *otcC* (uses NADPH/O_2) to form 5a, 11a-Dehydrotetracycline (5-DHTC, **Fig 1.10k-l**, Binnie *et al*., 1989; Peric-Concha *et al*., 2005). Bioinformatic analysis of OtcC indicated homology to bacterial flavin-type oxygenases. *OtcC* was disrupted by Peric-Concha *et al*., (2005) and analysis of secondary metabolites synthesised by the mutant led to the isolation of a novel 17C polyketide (**Fig1.11e**). The biosynthesis of a polyketide 2C shorter than the 19C OTC indicated that the product of *otcC* has an influence on the PKS to produce a nascent polyketamide of the correct length. It was therefore concluded that OtcC has a contributory role in the quaternary structure of the PKS complex (Peric-Concha *et al*., 2005).

5-DHTC is then hydroxylated at the C-5 position, to give 5a, 11a-Dehydrooxytetracycline (DHOTC) (**Fig 1.10m-n**). This step is exclusive to OTC biosynthesis. The terminal step of OTC biosynthesis involves the reduction of DHOTC to produce OTC (McCormick *et al*., 1962; Miller *et al*., 1965, **Fig 1.10n-l**). The enzyme complex of the dehydrogenase responsible for carrying out the terminal step of tetracycline biosynthesis in *S. aureofaciens* has been purified and sequenced (Novotna *et al*., 1990). This was found to be very similar to the gene product of *otcY2-4*, which was therefore putatively assigned as the terminal dehydrogenase (Thamchaipenet, 1994). The gene, *tchA*, from *S. aureofaciens* was recently isolated and characterized as the terminal dehydrogenase for the same step in the chlortetracycline biosynthetic pathway when it complemented a mutant blocked in this step but was mapped outside of the *ctc* cluster (Nakano *et al*., 2004). A homologue of *tchA* has been identified in *S. rimosus* using degenerate primers but biochemical analysis has not yet been completed (Zhang *et al*., 2006).

Some genes in the *otc* cluster remain uncharacterized. In addition to the putative

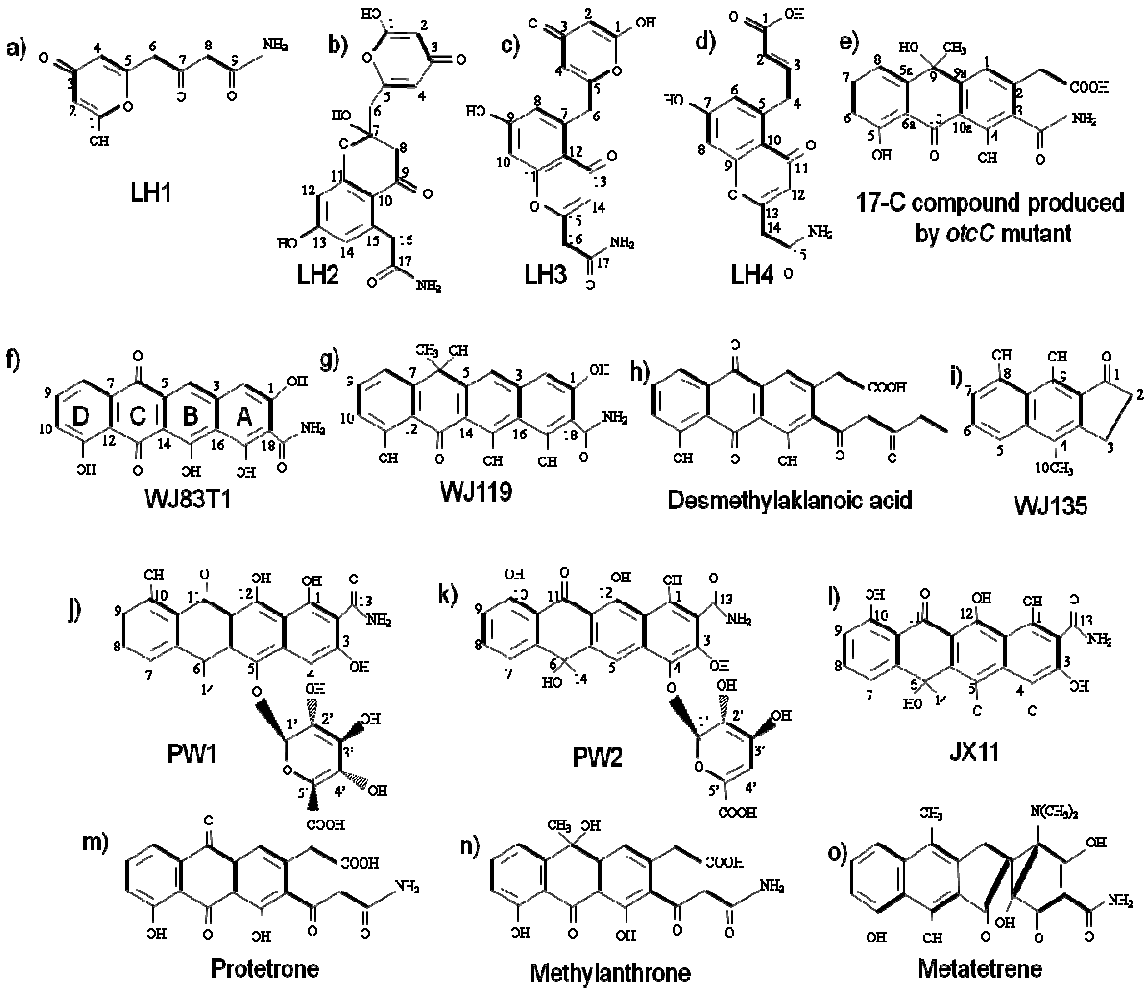


Fig 1.11 Novel polyketides synthesised by mutants disrupted at individual stages of OTC biosynthesis or isolated from heterologous co-expression of various *otc* genes

a)-d) LH-1 – LH-4; Novel, truncated polyketides isolated from *S. rimosus* mutant disrupted at *otcD1* (Petkovic *et al*., 1999); e) Novel 17C truncated polyketide isolated from Δ*otcC S. rimosus* mutant (Peric-Concha *et al*., 2005); f, g, h) Isolated from heterologous co-expression of *otc* genes in *S. coelicolor* CH999 (Zhang *et al*., 2007); i) Isolated from heterologous co-expression of *otc* genes in *S. coelicolor* CH999 (Zhang *et al*., 2008); j, k, l) Isolated from heterologous co-expression of *otc* genes in *S. coelicolor* CH999 (Wang *et al*., 2009); m, n, o) Shunt products isolated from mutants in tetracycline biosynthesis in *S. aureofaciens* (McCormick and Jensen, 1968; McCormick *et al*., 1968; McCormick *et al*., 1962). See text for details.

functions of *otcX3*, *otcD3*, *otcY2-2* and *otcY2-3,* the function of *otcD5* is unknown. It has a homologue ORF in the daunorubicin cluster of *S. griseus* (Krugel *et al*., 1993).

1.8 Regulation of Actinorhodin and Oxytetracycline biosynthesis

Regulation of the actinorhodin cluster is controlled primarily by the transcription of genes in the *actII* locus of *S. coelicolor* (Fernandez-Moreno *et al*., 1991), which consists of four ORF's. The product of *actII-ORF1* was assigned as a putative repressor protein, transcribed as a monocistronic mRNA, while *actII-ORF2* and *3* are co-transcribed and are believed to encode an antibiotic export complex (Caballero *et al*., 1991b). Overlapping, divergent promoters were found for each transcript in a similar fashion to bacterial tetracycline resistance mechanisms and the repressor/export transcriptional unit found in the *otc* cluster of *S. rimosus* (Caballero *et al*., 1991b; McDowall *et al*., 1991). Studies in *E. coli* using the *xylE* (catechol dioxygenase) reporter gene revealed that expression of *ORF1* strongly repressed the promoter of *ORF's 2/3* as well as its own (Caballero *et al*., 1991b).

The transcripts of *actII-ORF2* and *ORF4* (as well as the pathway activator gene for undecylprodigiosin) both contain a leucyl TTA codon, the tRNA for which (UUA) is encoded by *bldA* (Lawlor *et al*., 1987; Leskiw *et al*., 1991; Fernandez-Moreno *et al*., 1991). When a point mutation was introduced, changing the TTA codon to the more prevalent TTG, the abolition of actinorhodin biosynthesis normally observed in *bldA* mutants was reversed, illustrating the developmental control that *bldA* has over both initiation of and self-protection against actinorhodin biosynthesis (Fernandez-Moreno *et al*., 1991). S1-nuclease protection assays confirmed that transcription levels of *actII-ORF4* increased dramatically from very low levels during exponential growth to very high levels during the transition to stationary phase in *S. coelicolor*, co-inciding with an increase in transcription levels of *actIII* and *actVI-ORF1*, and leading to the production of actinorhodin (Gramajo *et al*., 1993).

Disruption of *actII-ORF2* delayed but did not abolish actinorhodin biosynthesis. This led to the hypothesis that the product of *ORF3*, co-transcribed with *ORF2* may have a more significant role in actinorhodin export or that another gene(s) existed that

encoded ACT resistance (Fernandez-Moreno *et al*., 1991). The phenotypes of the *bldA*, *actII-ORF4* and *actII-ORF2/3* mutants were very similar, illustrating the control of BldA over the biosynthesis of actinorhodin (Fernandez-Moreno *et al*., 1991).

ActII-ORF4 was characterized biochemically as a specific DNA-binding protein, as well as a positive transcription factor for specific ACT biosynthetic genes (Arias *et al*., 1999). *ActIII* (early-stage C-9 ketoreductase) was only transcribed when a mutated *actII-ORF4* was complemented with the wild-type (Hallam *et al*., 1988). ActII-ORF4 was later shown to bind to the intergenic regions between the transcriptionally divergent 'early' genes, *actI-ORF1* and *actIII* and with higher affinity to the 'late' genes *actVI-ORFA* and *actVI-ORF1* and initiate transcription in both. This confirmed ActII-ORF4 as a member of the Streptomycete Antibiotic Regulatory Protein family (SARP) (Wietzorrek and Bibb, 1997; Arias *et al*., 1999). Transcription of *actII-ORF4* was found to be regulated by the transcription factor, AtrA (actinorhodin-associated transcription factor A) to some degree, (Uguru *et al*., 2005). Disruption of *atrA* reduced the rate of transcription of *actII-ORF4* and reduced the rate of ACT biosynthesis but that of undecylprodigiosin and calcium-dependent antibiotic appeared to be unaffected (Uguru *et al*., 2005).

Most Type II PKS clusters are believed to be regulated by a homologue of *actII-ORF4* (Gramajo *et al*., 1993) e.g. *dnrI* from the daunorubicin cluster in *S. peucetius* (Stutzman-Engwall *et al*., 1992). However, a homologue within the *otc* cluster has yet to be identified. Transcription of *otcC* was found to be limited by high concentrations of phosphate, thus providing the first evidence of regulation of OTC production. OTC production was stimulated only in conditions of limited phosphate, indicating control of a specific transcription factor (Behal *et al*., 1982).

OTC biosynthesis was stimulated during the transition from the exponential to stationary phase of growth (McDowall *et al*., 1999), as observed for ACT biosynthesis (Gramajo *et al*., 1993). Promoters mapped in the intergenic regions between *otcX1/otcC* of the *otc* cluster, had conserved hexanucleotide sequences at the -10 and -35 regions (McDowall *et al*., 1999). Tandem repeats of these hexanucleotide sequences were found at positions -46 and -23 in the promoter

regions of both *otcX1* (*otcXp1*) and *otcC* (*otcCp1*) (McDowall *et al*., 1999). Tandem repeats were also found in the intergenic regions of the only other diverging gene pair in the cluster, *otcY2-1* and *otcY2-2* (McDowall *et al*., 1999). The spacing of these tandem repeats was found to be very similar to the DNA binding sites of the OmpR family of transcription factors, of which the recently established SARP family of proteins are related (Martinez-Hackert and Stock, 1997; Wietzorrek and Bibb, 1997). Furthermore, transcripts from these regions were found to increase dramatically at the onset of OTC production in conditions of limited phosphate. Thus, these results provided evidence that a protein similar to ActII-ORF4 or DnrI is involved in activating OTC biosynthesis at the transcriptional level, but the gene for which is not located in the *otc* cluster itself (McDowall *et al*., 1999).

1.9 Type I PKS biosynthesis: Erythromycin A

Type I PKS' are responsible for the biosynthesis of many bacterial, fungal and plant polyketides. They are characterized by their large multi-domain modules, as opposed to the dissociated complexes of proteins formed by Type II PKS'. The paradigm is the complex macrolide antibiotic, erythromycin A by *Saccharopolyspora erythraea*. Long, single polypeptides are synthesised, which fold into a quaternary structure organised as a trio of single multi-domain polypeptides.

Each polypeptide has similar catalytic properties (Cortes *et al*., 1990). These large ORF's were named *eryAI, II* and *III*, located within a 33kb segment of DNA (Donadio *et al*., 1991; 1992). The resistance gene, *ermE* was identified (Thompson *et al*., 1982a; Bibb *et al*., 1986), the product of which encodes resistance by catalysing N^6-dimethylation of 23S rRNA at a single site (Thompson *et al*., 1982b). 'Chromosome walking' was then used to identify the biosynthetic genes. The cluster was thus characterized as a single multi-functional enzyme complex with the *ermE* resistance gene located amongst the biosynthetic genes (Cortes *et al*., 1990).

Each ORF was found to encode two 'modules'. These modules fold to form functional synthase units (SU) that perform a similar sequence of enzyme modifications to those found in Type II PKS/FAS clusters. The starter unit for erythromycin A biosynthesis is not a standard metabolic derivative like acetyl- or

malonyl-CoA, but is instead propionyl-CoA as observed in Type II daunomycin biosynthesis in *S. peucetius* (Kitamura *et al*., 1981). This is followed by the condensation of six 3C methylmalonyl-CoA units to produce a series of 2C extensions towards the biosynthesis of the first isolatable intermediate, 6-deoxyerythronolide B (6-DEB). 6-DEB is a 14C macrolactone ring from which erythromycin A and its relatives are formed via further hydroxylation, glycosidation and methylation steps (Cortes *et al*., 1990).

The first acyltransferase (AT) domain loads the propionyl-CoA starter unit onto the ACP of the first SU followed by the transfer of the propionyl thioester to the active site of the first KS domain. The second ACP domain of the first SU is then loaded with a methylmalonyl-CoA extender unit by a second acyltransferase domain, which is then condensed and reduced by ketosynthase (KS) and ketoreductase (KR) domains, respectively, with the loss of CO_2. This yields a 5C intermediate, thus completing the first 2C extension of the propionyl primer (Katz and Donadio, 1993). This 5C intermediate is subsequently transferred to the KS of the second SU to act as the primer for the condensation of another methylmalonyl-CoA extender unit. Condensation of this unit forms a 7C intermediate. This process is repeated on the third SU without ketoreduction to give a 9C intermediate. The 4th SU differs to its partner SU (3rd) as it contains a peptide sequence homologous to ketoreductases, but does not contain the same catalytic residues as the other KR domains on the synthase. This led to the conclusion that this particular KR-like domain functions in a co-operative, non-catalytic capacity (Donadio *et al*., 1991). The 4th SU also contains dehydrogenase (DH) and enoyl reductase (ER) domains. These domains introduce a methylene group at C-7 of the DEB precursor. The 4th module produces an 11C intermediate that is elongated to a 13C intermediate by the 5th SU. At the 6th module, the final condensation steps are catalysed followed by cleavage of the 15C chain by a thioesterase domain, before its modification into the 15C 6-DEB (Hopwood and Khosla, 1992). This process is illustrated in **Fig 1.12a**.

Upon formation of the cyclised DEB, C-6 is hydroxylated followed by the addition of the deoxy-glucose derivatives mycarose and desosamine, by condensation at the hydroxyl groups of C-3 and C-5, respectively. Subsequent methylation of the condensed mycarosyl group and hydroxylation at C-12 of DEB produces

erythromycin A. Other relatives of ErA can be formed depending on the methylation status of the mycarose unit and the hydroxylation status of C-12. These are shown in **Fig 1.12b.**

Type I PKS' such as the EryA megasynthase are ideal candidates for genetic manipulation. Since each active domain catalyses a single step in the biosynthesis of the 15C-DEB precursor, introduction of a mutation in any catalytic domain should affect the final structure only at the point of mutation. This hypothesis was tested when a mutation was induced at the KR region of module 5 on *eryAIII* (Donadio *et al*., 1991). As predicted, the mutant produced the hypothesized structure, 5,6-dideoxy-3a-mycarosyl-5-oxoerythronolide B (**Fig 1.12c**), indicating that the 6th SU was able to use the altered intermediate as a substrate. The production of this structure indicated several points: the ketone group at C-5 remained unreduced as a result of the KR disruption; the absence of a hydroxyl group meant that glycosylation by desosamine did not occur; hydroxylation at C-6 did not occur; the post-15C DEB precursor modifying enzymes were flexible as they could still act without the hydroxyl groups at C-5 and C-6.

In theory, this suggested that many novel hybrid polyketides of potential therapeutic value could be synthesised depending on the mutagenic strategy employed (Donadio *et al*., 1992). The fact that polyketides were still produced by the mutants also suggested that the transfer of the growing polyketide chain between SU's was not dependent on the quaternary structure of the chain (Katz and Donadio, 1993). After the final synthetic step at DEBS-3, the intermediate is lactonised into DEB by a cyclase believed to be located 4kb downstream of DEBS-3 (Weber *et al*., 1991). Each ORF is responsible for a distinct stage in the biosynthesis of the 15C DEB precursor, therefore each polypeptide is referred to as either DEB Synthase 1, 2 or 3 (DEBS-1, 2 or 3).

There are various studies on the genetic manipulation of the Type I DEBS synthases (Kim *et al*., 2004; Chen *et al*., 2007). By combining their flexible catalytic properties with those of Type II PKS genes into hybrid constructs, expression in heterologous hosts such as *S. lividans* has produced compounds that are prototypic examples of strategic combinatorial biosynthesis of novel potentially-therapeutic polyketides

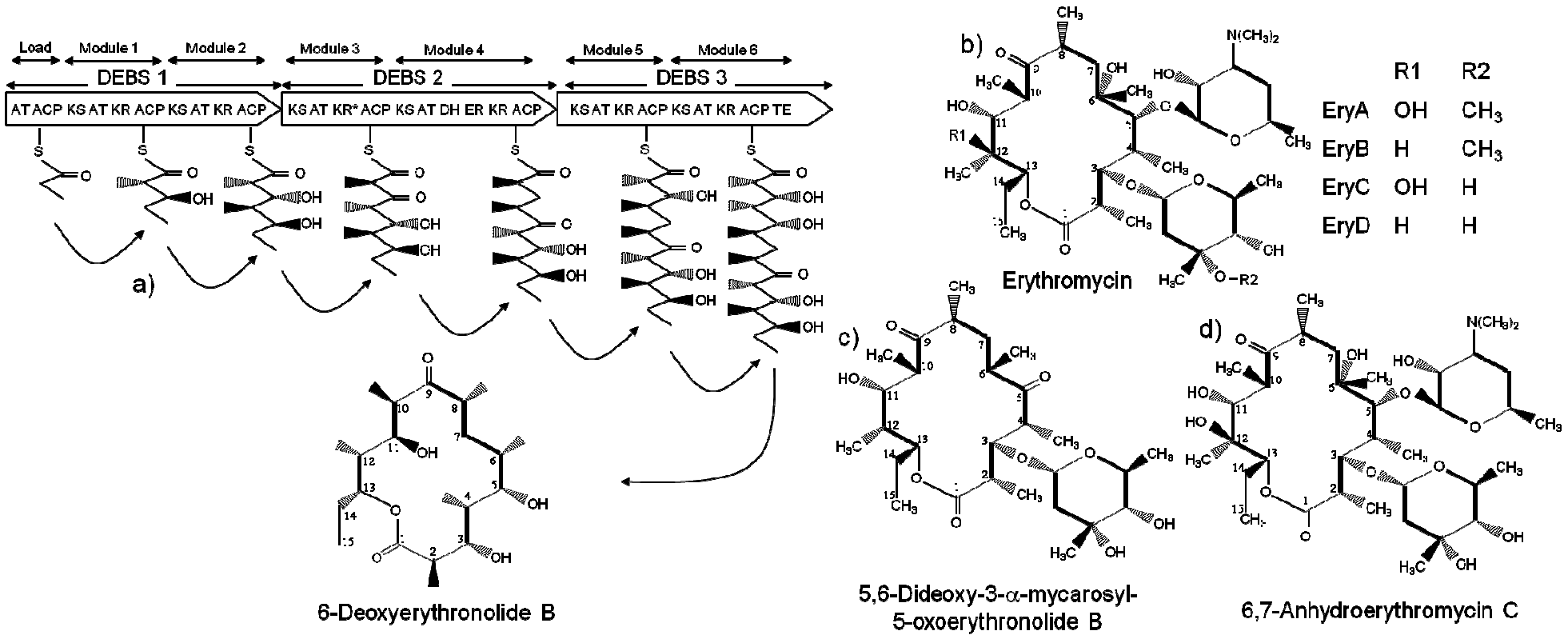


Fig 1.12 The biosynthesis of Erythromycin A and derivatives by Type I PKS

a) The biosynthetic pathway See text for details (Adapted from Donadio *et al.*, 1991).

b) the general structure of the erythromycin family, with variations in R groups between ErA-D

c) structure produced by mutant disrupted in KR of module 5 (see text for details)

d) 6,7-anhydroerythromycin C (Adapted from Katz and Donadio, 1993).

(Tang *et al*., 2000). Type I PKS' are flexible in their ability to produce 'unnatural polyketides' and libraries of compounds have been documented resulting from their manipulation (McDaniel *et al*., 1999). Recent reviews summarising advances in the manipulation of PKS systems are Gokhale *et al*., (2007) and Van Lanen and Shen, (2008). Combinatorial biosynthesis of bioactive compounds is also found as a natural process in clusters for many compounds, for example the biosynthesis of the antitimour drug, bleomycin in *S. verticillus* that is synthesised via a combination of a non-ribosomal peptide synthetase (NRPS) and a Type I PKS (Shen *et al*., 2001; 2002).

1.10 Type III PKS biosynthesis

Type III PKS' do not use a discrete acyl carrier protein to condense acyl units for biosynthesis of the polyketide but instead accept the acylated starter unit on to the synthase itself (Kreuzaler and Hahlbrock, 1975). This was demonstrated biochemically during a CHS assay when a malonyl-ACP intermediate was unable to replace malonyl-CoA on the synthase complex (Schuz *et al*., 1983). Chalcone synthase (CHS) in plant metabolism is the paradigm Type III PKS (**Fig 1.13a**). It is an ideal example of how products from Type III PKS' are often used as units for the synthesis of other compounds e.g. flavanoids, flavanols, flavanones, isoflavonoids and anthocyanins (Austin and Noel, 2003). Type III PKS were previously thought to be exclusive to plants, but the *S. coelicolor* A3(2) genome sequencing project led to the identification of at least three Type III PKS clusters (Bentley *et al*., 2002). These clusters have now been found throughout the Actinomycetes (Moore and Hopke, 2001; Moore *et al*. 2002).

CHS accepts 4-coumaryl-CoA as the starter unit directly on to the active site cysteine residue but also has substrate flexibility in accepting butyryl- and hexanoyl-CoA (Schuz *et al*., 1983). 4-coumaryl-CoA is synthesised by enzymatic modifications to phenylalanine from the shikimate pathway. This is followed by the iterative decarboxylative condensation of three malonyl-CoA units (Kreuzaler and Hahlbrock, 1975; Hrazdina *et al*., 1976; Schuz *et al*., 1983; Kreuzaler *et al*., 1978). As many as 12 CHS-like proteins have been discovered leading to the hypothesis that Type III PKS' are widespread in bacteria (Moore and Hopke, 2001).

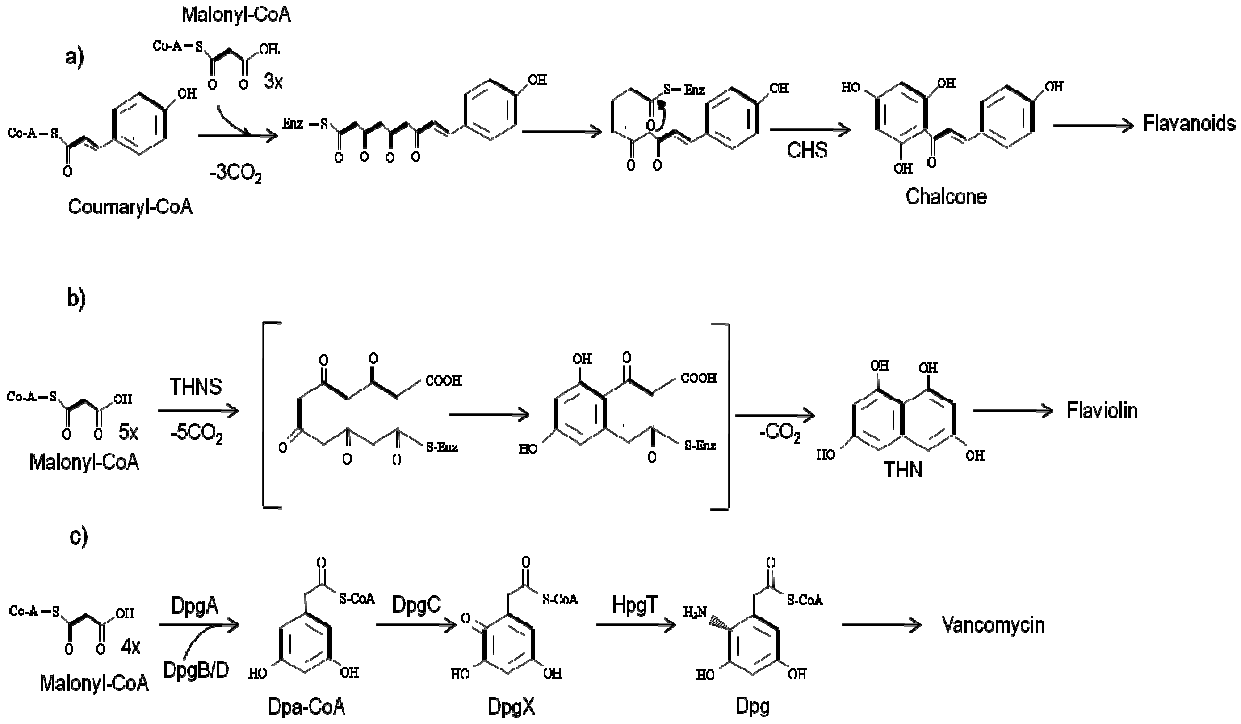


Fig 1.13 The biosynthetic pathways of chalcone, THN and Dpg

a) Biosynthesis of chalcone *via* condensation of a coumaryl starter unit and 3 malonyl units

b) Biosynthesis of THN via condensation of 5 malonyl units

c) Biosynthesis of Dpg via the Dpg operon of which, DpgA is a Type III PKS that condenses 4 malonyl units to form Dpa-CoA with assistance from DpgB/D before subsequent oxidation by DpgC and transamination by HpgT to form Dpg, which is then incorporated into the vancomycin scaffold.
See text for details.

Of the currently known bacterial Type III PKS', the first to be characterized was from *S. griseus* and synthesised the compound 1, 3, 6, 8-tetrahydroxynaphthalene (THN) (**Fig 1.13b**) catalysed by RppA (Funa *et al*., 1999; 2002). There is a homologue of *rppA* in *S. coelicolor* A3(2) (Moore and Hopke, 2001). Another example of *Streptomyces* Type III PKS' is the plant-like biosynthesis of benzoyl-CoA from phenylalanine as the starter unit in enterocin biosynthesis in *S. maritimus*, which is very similar to that of chalcone (Moore *et al*., 2002).

Type III PKS' make key components of glycopeptide antibiotics in *Amycolatopsis* spp. e.g. the synthesis of the non-proteinogenic amino acid, (*S*)-3, 5, dyhydroxyphenylglycine (Dpg) (**Fig 1.13c**). Dpg is synthesised by four proteins, DpgA-D of which DpgA is the Type III PKS involved in synthesising (*S*)-3,5-dihydroxyphenylacetyl-CoA (Dpa-CoA) that is subsequently modified to form Dpg, which forms an important component of the vancomycin scaffold (see Chen *et al*., 2001; Tseng *et al*., 2004; Pfeifer *et al*., 2001; Li *et al*., 2001).

1.11 Project Aims

The aim of the work in this thesis was to determine the role of the gene, *otcX3*, in the biosynthesis of oxytetracycline in *S. rimosus* using the following experimental strategies:

- Amplify and clone *otcX3* into pET-driven expression vectors
- Over-expression of *otcX3* in *E. coli*
- Isolation of recombinant OtcX3 from *E. coli* using His•tag affinity-purification
- Investigate the ability of recombinant OtcX3 to bind various acyl thioesters units used as primers in the biosynthesis of Type II PKS' e.g. acetyl-, malonyl-, malonamyl-CoA
- Disrupt *otcX3* on the chromosome of *S. rimosus* M4018 and analysis of metabolites produced by the mutant

Chapter 2:

Materials and Methods

2.1 General Materials

2.1.1 *E. coli* strains

Designation	Description / Genotype	Source
DH5α	*sup*E44, Δ*lac*U169, (ϕ80$\lambda\alpha_{\lambda}$*Z*ΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1	(Sambrook *et al.,* 1989)
ET12567	F$^-$ *dam*13::Tn*9, dcm*6, *hsd*M, *hsd*R, *rec*F143::Tn*10, gal*K2, *gal*T22, *ara*14, *lac*Y1, *xyl*5, *leu*B6, *thi*1, *ton*A31, *rps*L136, *his*G4, *tsx*78, *mtl*1 *gln*V44	(MacNeil *et al*., 1992)
BW25113	Δ(*ara*D-*ara*B)567, Δ*lacZ*4787(::rrnB–4), *lac*Ip-4000(*lac*I^{Q}), λ$^-$, *rpo*S369(Amp), *rph*-1, Δ(*rhaD-rhaB)*568, *hsd*R514	(Datsenko and Wanner, 2000)
DS941	*rec*F143, *pro*A7, *str*31, *thr*1, *leu*6, *tsx*33, *mt*12, *his*4, *arg*E3, *lac*Y+, *lacZ*ΔM15, *lac*I^{q}, *gal*K2, *ara*14, *sup*E44, *xyl*5	(Hunter, personal comm.)
JM109	F$^{'}$ *tra*D36 *pro*A^{+}B^{+} *lac*I^{q} Δ*(lac-pro*AB*)* *gln*V44 *e*14^{-} *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi hsd*R17	(Yanisch-perron *et al*., 1985)
AB2834 (28)	*aro*E *mal thi*	(Hunter, personal comm.),
AB2848 (29)	*aro*D352 *tsx*-356 *sup*E42 *aro*D	"
AB2834::Tn*10*(53)	Isogenic partner to AB2834, Tn*10*	"
AB2848::Tn*10*(54)	Isogenic partner to AB2834, Tn*10*	"
DS883	Source of Tn*10*	"
DS948	"	"
NovaBlue	*end*A1 *hsd*R17 ($r_{K12}^{-}m_{K12}^{+}$) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F` *pro*A^{+}B^{+} *lac*I^{q}ZΔM15::Tn*10*]	Novagen
HMS174::DE3	F^{-} *rec*A*1 hsd*R (rK_{12}^{-} mK_{12}^{+}) Rif^{R} (DE3)	Novagen (Studier and Moffat, 1986)
BL21::DE3	F^{-} *omp*T *hsd*S_B (r_B-m_B-) *gal dcm* (DE3)	Novagen (Studier and Moffat, 1986)
BL21/pLysS::DE3	" pLysS	"
Rosetta::DE3	F^{-} *omp*T *hsd*S_B ($r_B^{-}m_B^{-}$) *gal dcm lac*Y1 (DE3) pRARE(Cmp^{R})	Novagen
Origami B::DE3	F^{-} *omp*T *hsd*S_B ($r_B^{-}m_B^{-}$) *gal dcm lac*Y1 *gor*522::Tn*10 trx*B::kan (DE3)	Novagen
Tuner::DE3	Derivative of *E. coli* BL21: F^{-} *omp*T *hsd*S_B ($r_B^{-}m_B^{-}$) *gal dcm lac*Y1 (DE3)	Novagen

2.1.2 *E. coli* and *Streptomyces* vectors

Designation	Antibiotic resistance / Description	Source/ Reference
pSRG3	Ap^R; 5.2kb wild-type *Pst*I fragment from *otcX/otcC/otcZ* region of *otc* cluster ligated into pIBI24 (Dente *et al*., 1983)	Garven, 1995, **App. A. 7**
pIJ790	Cmp^R - temp. sensitive replicon (30°C) contains λ Red genes; *gam, bet, exo*	Gust *et al*., 2002; 2003, **App. A. 8**
pIJ773	$Apra^R$; contains *aac(3)*IV-*oriT* cassette	Gust *et al*., 2002; 2003, **App. A. 9**
pT7Blue3/ *otcX3*	Ap^R/Kan^R; 1.026kb *Nde*I - *Bam*HI PCR product of *otcX3* from *otc* cluster blunt-ligated into pT7Blue3 Eco RV site to form pT7Blue3/*otcX3*.	This study / Novagen (**Chapter 3)**
pET15X3	Ap^R *lac*I; N-terminal His•tag®, pET T7 expression system, 1.026kb *Nde*I-*Bam*HI fragment from pT7Blue3/*otcX3* ligated into open pET15b	Novagen, **App. A. 5**
pET33X3	Kan^R *lac*I;N-terminal his•tag®, T7•tag®, pET T7 expression system, 1.026kb *Nde*I-*Bam*HI fragment from pT7Blue3/*otcX3* ligated into open pET33b(+)	Novagen **App. A. 6**
pBR322	Ap^R Tc^R; ColEl Ori	(Bolivar *et al*., 1977) **App. A. 13**
EH1	(Supercos1 derivative) Cosmid isolated from *S. rimosus* M4018 cosmid library. Contains entire *otc* cluster. Ap^R, Kan^R	(Almuteirie, personal comm.)
AH6	As EH1 but does not contain entire *otc* cluster	"
EH1 Δ*otcX3::aac (3)IV-oriT*	Shuttle vector created by replacement of *otcX3* on EH1 with *aac(3)IV-oriT* cassette Ap^R, Kan^R, $Apra^R$	**Chapter 4**
AH6 Δ*otcX3::aac (3)IV-oriT*	Shuttle vector created by replacement of *otcX3* on AH6 with *aac(3)IV-oriT* cassette Ap^R, Kan^R, $Apra^R$	**Chapter 4**
pATX3	Derivative of pSET152; 1.1kb *Eco*RI Tsr^R fragment and 350bp *Eco*RI/*Nde*I *erm*E*tuf1 fragment ligated into *Eco*RI site (Tilley, personal comm.). *OtcX3* ligated on *Nde*I-*Bam*HI fragment cut from pET33X3	Bierman *et al*., (1992), **App. A. 12**
pG2	shuttle vector, $Thio^R$, $Apra^R$	(Almuteirie, personal comm.), **App. A. 11**

2.1.3 *Streptomyces* strains

The prototrophic strain *S. rimosus* M4018, developed by Pfizer was the strain used in this work (Rhodes *et al*., 1981).

2.1.4 Methicillin-resistant *Staphylococcus aureus* (MRSA) strains

Several strains of community-acquired MRSA were used and discussed in **Section 4.3.9** of this work (Smith, K. personal comm.); however, sensitivity to tetracycline and information on other known antibiotic resistances were the only known characteristics.

2.1.5 Enzymes

For PCR, Red T_{aq} Polymerase (Sigma), T_{aq} Polymerase (Fermentas) and Expand Hi-Fidelity DNA polymerase (Roche) were used accordingly. For ligations, T_4 DNA ligase (Bioline, Fermentas, Promega) was used accordingly. Klenow enzyme (Roche) was used in ligations or for the random-priming of DNA probes for hybridization, as required. For analysis of DNA, the following restriction enzymes were used; *Nde*I, *Bam*HI, *Hin*dIII, *Eco*RI, *Sac*I and *Pst*I (New England Biolabs, Invitrogen, Fermentas, Promega). Reaction conditions for each enzyme were used as described by protocols provided by the respective manufacturer.

2.1.6 Kits and appliances

The following kits/applications were used during this work: cloning of PCR products - Blunt Cloning Kit® (Novagen); purification of DNA fragments from agarose gels - QIAEX Gel Extraction kit (Qiagen) or Wizard® SV Gel and PCR clean-up System (Promega); isolation of plasmid DNA from *E. coli* - Wizard® Plasmid Prep (Promega); hybridization - Hi-Bond-N nitrocellulose membranes (Amersham) and DIG-DNA Labelling Kit (Roche) for preparation of probe; for calibration of gel filtration column – Protein Calibration Kit (Sigma). All kits used according to

protocols provided by the respective manufacturers. An automated Flow Pressure Liquid Chromatography (FPLC) appliance from Amersham Biosciences Ltd. was used to purify over-expressed recombinant protein. His•tag purification, gel filtration columns and G-200 sephadex resin were purchased from Amersham Biosciences Ltd. Micro-concentrators for the concentration of protein samples were purchased from Pall Corporation.

2.1.7 Sources of chemicals, media, antibiotics and standard markers

All reagents for preparation of growth media were purchased from Oxoid Ltd., UK or Difco. Soya bean flour was obtained from a local organic health store. Analytical Reagent (AnalaR) grade chemicals used for the preparation of solutions/media were purchased from Sigma-Aldrich Co., Poole, UK, Gibco BRL Ltd, Paisley, BDH Chemicals, Poole, UK, Fischer Chemicals UK Ltd, VWR International Ltd Laboratories or Difco, unless stated. All antibiotics were supplied by Sigma-Aldrich Co., UK. λ*Hin*dIII DNA, 1Kb and 100bp DNA markers were purchased from Promega Corporation or Fermentas. Protein standard markers were purchased from either Promega Corporation or BioRad and prepared for use according to protocols described by the respective manufacturers.

2.2 Standard General Methods

2.2.1 Sterilisation

Growth media and all chemical solutions were sterilised by autoclaving at 121^{o}C for 20 minutes unless otherwise stated. Stock solutions of antibiotics and heat-labile solutions e.g. amino acids were filter-sterilised through a 0.22μm pore membrane.

2.2.2 Media for culturing *E. coli*

L Broth (agar): 10g tryptone, 5g yeast extract, 10g NaCl per 1L dH_2O (addition of 20g agar technical no. 3 for agar); adjusted to pH 7.2 with 0.1M NaOH

Terrific Broth: 12g tryptone, 24g yeast extract and 8ml 50% glycerol per 1L dH_2O; adjusted to pH7.2 with 0.1M NaOH. 10ml sterile phosphate buffer (0.231g KH_2PO_4, 1.254g K_2HPO_4) added immediately before use.

SOC: 20g tryptone, 5g yeast extract, 0.5g NaCl and 10ml 250mM KCl per 1L dH_2O; adjusted to pH 7.2 with 0.1M NaOH. Sterile $MgCl_2$ and glucose were added immediately before use to final concentrations of 10 and 20 mM, respectively.

2.2.3 Media and buffers used for growth of *Streptomyces rimosus* M4018

Tryptone Soya Broth (TSB): 30g Oxoid Tryptone Soya Broth powder per 1L dH_2O.. Sterile 2.5M $MgCl_2.6H_2O$ was added to a final concentration of 5mM immediately before use. For isolation of total DNA, sterile glycine was added to a final concentration of 0.5% w/v.

Emerson's agar: 4g beef extract, 1g yeast extract, 4g peptone, 10g glucose, 2.5g NaCl, 10g agar technical no. 3 per 1L dH_2O; adjusted to pH 7.2 with 0.1M NaOH.

CRM: 10g glucose, 103g sucrose, 10.12g $MgCl_2.6H_20$, 5g yeast extract and 15g Oxoid TSB per 1L dH_2O (Pigac and Schrempf, 1995).

Soya Mannitol Agar (SFM): 20g mannitol, 20g soya bean flour, 20g agar technical no. 3 per 1L dH_2O; processed through autoclave twice before use.

2.2.4 Selective concentrations of designated antibiotics

Antibiotic	Solvent	Stock conc. ($mg.ml^{-1}$)	Final conc. in *E. coli* ($\mu g.ml^{-1}$)	Final conc. in *S. rimosus* ($\mu g.ml^{-1}$)
Ampicillin	dH_2O	50	100	Not used
Apramycin	"	100/400	100	400
Kanamycin	"	25/200	25	>250
Gentamycin	"	100	Not used	>250
Erythromycin	"	20	"	20-40
Chloramphenicol	Ethanol	25	25	Not used
Tetracycline (light-sensitive)	"	10	12.5	Not used
Thiostrepton (light-sensitive)	DMSO	10	Not used	10

Antibiotics were added to molten media at 50°C. Light-sensitive antibiotics were stored accordingly in darkened containers. All antibiotics were stored at -20°C with the exception of thiostrepton, which was stored at 4°C.

2.2.5 Preparation of spore suspensions of *S. rimosus* strains

An SFM plate was inoculated with a single colony of *S. rimosus*. Strains were grown at 30°C for several days under antibiotic selection when appropriate to a stage of sporulating, confluent growth. Concentrated spore suspensions were prepared as outlined by Hopwood *et al*., (1985) and stored at -20°C in aliquots of 1ml in sterile dH_2O for short-term or 20% sterile glycerol for long-term storage.

2.2.6 Conditions for growth of *E. coli*

Strains of *E. coli* were normally cultured in L Broth with antibiotic selection when appropriate (**Section 2.2.4**). Cultures were grown typically overnight in standard media at 37°C, 250 rpm, unless otherwise stated. To maximise aeration and facilitate growth cultures grown using a liquid to volume ratio equal to or less than 1:5.

2.2.7 Conditions for growth of *S. rimosus*

Strains of *S. rimosus* were grown at 30°C, 250rpm in Erlenmeyer flasks for 2-3 days or until stationary phase was reached in TSB for isolation of total DNA or in CRM for preparation of electro-competent mycelium (Pigac and Schrempf, 1995). Normally, 100µl concentrated spore suspension was used to inoculate 100ml medium; however, empirical adjustments were often required to obtain a working inoculum. A medium to flask volume ratio of 1:5 was never exceeded, as for *E. coli*.

2.2.8 Preservation of strains

Glycerol stocks were used to preserve *E. coli* strains. An overnight culture of the strain was grown in standard media with antibiotic selection, when appropriate. The overnight culture was then added to a 2ml cryotube with 80% glycerol (50% glycerol final concentration) and mixed. Stocks were frozen at -70°C. To retrieve the strains, a scraping from the stock was taken aseptically and spread onto an agar plate for single colonies, which would subsequently be used for further inoculation.

For the preservation of *S. rimosus* strains, an SFM slope (made by pouring molten SFM agar into a sterile boiling tube angled at 5° to the horizontal) was inoculated with a single colony of the *S. rimosus* strain and grown for approximately 4-5 days or until maximum sporulation was observed. Upon mature growth, slopes were sealed and stored at -20°C indefinitely.

2.2.9 Preparation of *E. coli* strains rendered competent by $CaCl_2$

An overnight culture of the strain was used to inoculate an appropriate volume of pre-warmed (37°C) L broth (100ml for small-scale or 1L for large-scale preparations) using a dilution factor of 1:100. The flask was incubated at 37°C, 250rpm, until exponential growth was reached, indicated by an optical density at 600nm ($O.D._{600}$) between 0.4 and 0.8. Cells were harvested by centrifugation at 3,000g, 4°C for 10 minutes and the supernatant discarded. The pellet was gently resuspended in 1/5

volume of ice-cold 100mM $MgCl_2$ and incubated on ice for 5 minutes. The cells were pelleted at 3,000g/4^{o}C for 10 minutes, the supernatant discarded and the pellet resuspended gently in 1/5 volume ice-cold 100mM $CaCl_2$. The suspension was incubated on ice for at least 15 minutes before harvesting the cells again at 3,000g/4^{o}C, 5 minutes and the supernatant discarded. The cells were resuspended gently in 2ml 100mM $CaCl_2$/20% glycerol and divided into 100µl aliquots, quick frozen in liquid nitrogen and stored at –70^{o}C. Aliquots were subsequently thawed on ice when required for transformation.

2.2.10 Preparation of *E. coli* strains for electroporation

E. coli strains were grown as described in **Section 2.2.9**. The cells were chilled on ice for 30 minutes and subsequently harvested at 3,000g, 4^{o}C, 10 minutes and the supernatant discarded. The cells were resuspended gently in 50ml ice-cold 10% glycerol per 50ml harvested culture, pelleted by centrifugation and the supernatant discarded. This was repeated twice further with 25 and 10ml ice-cold 10% glycerol to remove as much salt as possible. The cells were resuspended in 1ml ice-cold 10% glycerol, divided into aliquots, quick frozen and stored at -70^{o}C, as described in **Section 2.2.9**.

2.2.11 Preparation of *S. rimosus* strains for electroporation

The method described by Pigac and Schrempf (1995) was followed. CRM containing glycine (0.5% final conc.) was inoculated with concentrated *S. rimosus* M4018 spore suspension (**Section 2.2.7**) and grown at 30^{o}C, 250rpm for 24 hrs, harvested at 12,000g at 4^{o}C for 10 minutes and the supernatant discarded. The mycelium was washed in 50ml ice-cold 10% (w/v) sucrose and pelleted as before. The pellet was then resuspended and washed in 50ml ice-cold 15% (v/v) glycerol and pelleted again. The mycelium was resuspended in 10ml 15% (v/v) glycerol containing 100µg.ml^{-1} lysozyme and incubated at 37^{o}C, 30 minutes and pelleted followed by resuspension in ice-cold 15% (v/v) glycerol twice further. The mycelium was finally resuspended in 1-5ml (depending on viscosity) electroporation solution; 30% (w/v) PEG_{1500}, 10%

(v/v) glycerol and 6.5% (w/v) sucrose. The suspension was divided into aliquots (50µl), ready for immediate use or quick frozen and stored -70^{o}C.

2.2.12 Transformation of *E. coli* strains by heat shock

1µl plasmid or cosmid DNA of known concentration was added and mixed gently with an aliquot of pre-prepared cells (**Section 2.2.10**). The suspension was incubated on ice for 1 minute before incubation at 42^{o}C for 30s to initiate the heat-shock response, followed by incubation on ice for a further 2 minutes. 1ml L broth was added to the suspension and incubated at 37^{o}C, 250rpm for 60-90 minutes to allow phenotypic expression. The cell suspension was plated out using serial dilutions onto L agar supplemented with antibiotic selection, when appropriate. The plates were incubated at 37^{o}C for a maximum of 16hrs and stored at 4^{o}C.

2.2.13 Transformation of *E. coli* strains by electroporation

Plasmid or cosmid DNA was gently mixed with 100µl electro-competent cells (**Section 2.2.12**). Electroporation was conducted with 0.1cm electro-cuvettes using a BioRad MicroPulser™ set to; 200Ω, 25µF and 2.5 kV with an expected time constant of between 4.5-4.9ms. 1ml ice-cold L broth was added immediately, followed by incubation at 37^{o}C, 250rpm for 60-90 minutes. The cell suspension was plated out as described in **Section 2.2.12**.

2.2.14 Transformation of *S. rimosus* strains by electroporation

The method was used as described by Pigac and Schrempf (1995). Cosmid or plasmid DNA of a known concentration isolated from a *dcm*$^-$ *dam*$^-$ strain of *E. coli* (ET12567, **Section 2.1.1**) was gently mixed with 50µl electro-competent *S. rimosus* M4018 (**Section 2.2.11**) and incubated on ice for 1 minute. Electroporation was conducted using 0.2cm electro-cuvettes and a BioRad MicroPulser™ set to; 400Ω (or up to 600Ω), 25uF, 2.0kV. After electroporation, 1ml CRM was added and the suspension was transferred to a Sterilin Falcon tube and incubated at 30^{o}C, 250rpm for at least 3 hours. The cell suspension was plated using serial dilutions as described before on TSA supplemented with antibiotic selection, when appropriate. After incubation at 30^{o}C for 48hrs colonies expressing antibiotic resistance were isolated and inoculated on to fresh TSA plates for further analysis.

2.2.15 Over-expression of *otcX3* in *E. coli*

λDE3 lysogenic strains of *E. coli* (**Section 2.1.1**) containing the *lacUV5* promoter designed for over-expression of genes using the pET expression system (**Section 3.2**, Studier and Moffat, 1986) were grown in L broth, as described in **Section 2.2.9**. 100ml L broth was normally used for small-scale and 1L for large-scale preparations. Cultures containing pET33X3 were induced at an O.D.$_{600}$ between ~0.6-0.8. The 100ml culture was aseptically split into two. One culture was induced with IPTG at a final concentration of 1mM and the other was left uninduced as a control for analysis by SDS-PAGE. A second control strain containing pET33b(+) with no insert was often used. 1ml samples were taken at hourly intervals, the cells harvested by centrifugation at 13,000g, 4^{o}C for 10 minutes, the supernatant discarded and the pellet either lysed immediately (**Section 2.4.4**) or stored at -20^{o}C.

2.3 DNA Methods

2.3.1 Isolation of vector DNA from *E. coli* by alkaline lysis

Reagents:

Solution I: 50mM glucose, 25mM Tris.Cl, 10mM EDTA, pH 8.0. Stored at room temperature.

Solution II: 0.5ml 4M NaOH, 0.5ml 20% (w/v) SDS, 9ml dH_2O. Not sterilised, made fresh.

Solution III: 60ml 5M potassium acetate, 11.5ml glacial acetic acid per 100ml dH_2O. Stored at room temperature.

DNase-free RNase: 10mM Tris, pH 7.8, 15mM NaCl. 10mg.ml^{-1} RNase was added, boiled for 15 minutes and cooled. Stored at –20^{o}C. Used at final concentration of 50μg.ml^{-1} RNase.

Neutral Phenol/chloroform: 5g phenol : 5ml chloroform : 1ml dH_2O. Not sterilised.

10x TE buffer: 100mM Tris, 10mM EDTA, pH8.0. Used at 1x concentration. Stored at room temperature.

The method used for isolating plasmid DNA was based on alkaline lysis, as described by Birnboim and Doly (1979). Typically, vector DNA was isolated from small cultures of *E. coli* (5-15ml).

Cultures of *E. coli* containing vector DNA grown to stationary phase were harvested by centrifugation at 13,000g for 30s. The pellet was fully resuspended in 100μl of ice-cold solution I and incubated on ice for 2 minutes. 200μl solution II solution was added and mixed gently by inversion. 150μl ice-cold solution III was added to the lysate and mixed by inversion until the white precipitate was evenly dispersed throughout the suspension and incubated on ice for 2 minutes before the white precipitate was pelleted at 13,000g, 10 minutes. The supernatant was transferred to a sterile 1.5ml Eppendorf tube, avoiding any transfer of the white precipitate. Vector DNA was extracted with an equal volume of neutral phenol/chloroform and mixed thoroughly. The mixture was then separated into organic and aqueous phases by

centrifugation at 13,000g, 5 minutes. Any transfer of white precipitate was negotiated by removal of the aqueous layer and re-extracted with a further volume of neutral phenol/chloroform.

The aqueous upper layer was transferred to a fresh, sterile 1.5ml Eppendorf tube. DNA was precipitated by 1 volume of isopropanol, incubated on ice for 10 minutes and pelleted at 13,000g, 10minutes. The supernatant was discarded and the nucleic acid pellet washed with 1ml 70% (v/v) ethanol and pelleted as before. The supernatant was decanted off followed by brief centrifugation to collect any residual, which was removed carefully and discarded. The pellet was left to air-dry for ~30minutes and resuspended in 1x TE buffer (typically 25-50µl) and incubated with 1µl 1mg.ml^{-1} DNase-free RNase (37^{o}C, 30 minutes). The DNA was stored at –20^{o}C.

2.3.2 Isolation of total DNA from *S. rimosus*

Reagents:

TEGLR buffer: 25mM Tris-HCl, 10mM EDTA, 50mM; to pH 8.0 using HCl. Lysozyme and pre-boiled DNAase-free RNAse added to final concentrations of 2mg.ml^{-1} and 50µg.ml^{-1}, respectively immediately before use.

Lysis solution: 0.3M NaCl, 2%SDS. Stored at room temperature. Not sterilised.

3M sodium acetate: unbuffered. Stored at room temperature.

Neutral phenol/chloroform: see **Section 2.3.1**.

10x TE buffer: see **Section 2.3.1**.

Cultures of *S. rimosus* were grown as described (**Section 2.2.7**). Mycelium were harvested in Oakridge tubes at 10,000g, 4^{o}C for 10 minutes and the supernatant discarded. The pellet was washed twice by resuspension in 30ml 10% (w/v) sucrose, pelleted and the supernatant discarded. The pellet was resuspended in 3ml TEGLR and incubated at 37^{o}C for 30-60min and gently mixed, periodically. 3ml 0.3M NaCl, 2% (w/v) SDS was added and mixed immediately by inversion. 6ml neutral phenol/chloroform was added, mixed by inversion and the organic/aqueous layers separated by centrifugation at 10,000g/4^{o}C, 10 minutes. The aqueous layer was

removed carefully and transferred to a fresh, sterile Oakridge tube. This was repeated until no white interface was observed (normally between 3 and 5 extractions). 5 ml choloroform was added and mixed by inversion, organic/aqueous layers separated by centrifugation and the aqueous layer transferred to a fresh tube. The DNA was precipitated in 1/10 volume 3M unbuffered Sodium Acetate and 2.5 volumes of isopropanol at -20^{o}C overnight. DNA was then pelleted at 10,000g, 4^{o}C, 30 minutes, washed with 1ml 70% (v/v) ethanol and pelleted. The supernatant was discarded and the pellet left to air-dry. The pellet was dissolved in 5ml 1xTE buffer at 4^{o}C (could take several hours) before 2 further neutral phenol/chloroform extractions/DNA precipitation steps. The DNA pellet was finally dissolved in a minimum volume 1x TE buffer and stored at 4^{o}C.

2.3.3 Precipitation of DNA

Precipitation of all DNA was carried out using 2-2.5 volumes of absolute ethanol in the presence of salt (1/10 volume using 5M NaCl or 3M sodium acetate) and incubated on ice for at least 2 hours but often overnight at -20^{o}C. Precipitated DNA was pelleted by centrifugation at 13,000g, 4^{o}C, for 30 minutes the supernatant removed and the pellet dried. The pellet was washed with 1ml 70% (v/v) ethanol before brief centrifugation and removal of ethanol to allow the pellet to dry.

2.3.4 Digestion of DNA by restriction enzymes

Digests were normally prepared in volumes of 20µl or up to 100µl for more concentrated DNA samples. Digests of vector DNA were incubated at 37^{o}C for up to 3 hours and a minimum of 4 hours for total DNA. On occasions when digestion by more than one enzyme was required, compatible enzymes (functioned in same buffer) could be used concurrently in the same reaction. When two separate reactions were necessary, precipitation of the DNA between reactions was required (**Section 2.3.3**).

2.3.5 Ligation of DNA fragments

Conditions for ligation reactions (**Section 2.1.5**) were determined in relation to the size and concentrations of both the insert and open vector. Insert and vector DNA was quantified from images of an agarose gel (**Section 2.3.10**). The following calculation was then used to establish the required ratio of compatible insert:vector: **Vector size/insert size x (½ x ng vector) x 3 = min ng insert required.** Reactions were typically prepared in final volumes of 20µl and incubated at room temperature for approximately 2 hours or overnight at 4^{o}C before transformation.

2.3.6 Separation of DNA fragments by agarose gel electrophoresis

Solutions:

Agarose gel loading buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% (w/v) Ficoll in dH_2O. Stored at room temperature. Not sterilised.

50x TAE buffer: 242g Tris, 57.1ml glacial acetic acid, 100ml 0.5M EDTA per 1L dH_2O, pH 8.0. Used at 1x concentration.

Digested samples of DNA were separated and analysed by agarose gel electrophoresis. For resolution of fragments between ~1-6 kb, 0.8-1% (w/v) agarose in 1x TAE buffer was used. For larger fragments, 0.4-0.5% (w/v) was used and for smaller fragments (<1kb), 1.2% (w/v) was sufficient. Molten agarose in 1x TAE (~50^{o}C) was poured into the mould and allowed to set, inserting a comb to create sample wells. Samples of DNA with 10% (v/v) loading buffer and Appropriate DNA markers (**Section 2.1.7**) were loaded appropriately, as required. Typically, DNA samples were separated at ~70-80V for 0.8-1% (w/v) agarose gels. For lower % (w/v) agarose, (~40-50V or 20-25V overnight).

Gels containing separated DNA fragments were bathed in a solution (approx. 200µg.l^{-1} final concentration) of ethidium bromide in dH_2O for 15-20 min, destained in dH_2O for 5-10 minutes. Alternatively, ethidium bromide was added to molten agarose before setting (approx. 100 µg.l^{-1} final concentration). Images of separated

DNA fragments were captured on a UV transilluminator at 302nm using software-based, automated digital photography. Saved images were enhanced using Adobe Photoshop when required. When necessary, DNA fragments were recovered using an appropriate purification kit (**Section 2.1.6**).

2.3.7 Sequencing of plasmid DNA

DNA was prepared at high concentrations in dH_2O using a DNA preparation kit (**Section 2.1.6**). Roth Tate, a technician at the University of Strathclyde, was responsible for the sequencing of plasmid DNA. Sequencing was conducted using an ABI-373 sequencer from Applied Biosystems based on the principles outlined by Sanger *et al*., (1977). Universal oligonucleotide sequencing primers, M13 or T7 were used and sequence data were analysed using the software program Geneious.

2.3.8 Amplification of *otcX3* by PCR

Amplification of *otcX3* using the plasmid, pSRG3 (**Section 2.1.2**, Garven, 1995) as the template DNA was conducted using Red T_{aq} Polymerase (Sigma, **Section 2.1.5**). Oligonucleotide primers were designed to complement the sequence of *otcX3* and synthesised by MWG, Germany. *Nde*I and *Bam*HI restriction sites were incorporated into the primers to facilitate cloning. PCR reactions were normally conducted in 0.5ml Eppendorf tubes. The annealing temperature for each primer was calculated as follows: **Tm(melting temp) = 59.9 + 1/2(G + C content %) – 600/length primer**.

Forward primer (lab stock 279) – *otcX3* forward sequence from pSRG3:
$^{5'}$ - GGG **CAT ATG** ACG GCG GAC ACG AAG GC - $^{3'}$ (*Nde*I site highlighted)
Reverse primer (lab stock 280) – *otcX3* reverse complement sequence from pSRG3:
$^{5'}$ – GG**G GAT CC**T CAG GGA ATC CGG TAC CCC – $^{3'}$ (*Bam*HI site highlighted)

Reaction/Cycle conditions:

Forward primer (100pmol);	1µl
Reverse primer (100pmol);	1µl
Template DNA (~25ng.µl^{-1});	1µl
10x Reaction buffer;	5µl
$MgCl_2$ (25mM);	5µl (2.5mM)
dNTPs (1.25mM);	8µl
DMSO;	5µl (10% (v/v) final concentration)
DNA polymerase (1U.µl^{-1});	1µl
dH_2O;	23µl
Total volume;	**50µl**

1: Hot start;	94^{o}C, 5 min (enzyme added after 4.5min)
2: Denaturation;	94^{o}C, 30s
3: Annealing;	57^{o}C, 30s
4: Elongation;	72^{o}C, 1min, 15s (repeat steps 2-4 x 29)
5: Denaturation;	94^{o}C, 1 min
6: Annealing;	57^{o}C, 1 min
7: Final elongation;	72^{o}C, 5min

2.3.9 Amplification of *aac(3)*IV-*oriT* cassette

Expand Hi-Fidelity Taq polymerase (Roche) (**Section 2.1.5**) was used to amplify the *aac(3)*-IV-*oriT* cassette using the plasmid pIJ773 (**Section 2.1.2**) as the template DNA. Oligonucloetide primers were designed to incorporate 39 nucleotide flanking regions (highlighted in bold) of *otcX3*, including start (ATG) and stop (TGA) codons on the chromosomal DNA of *S. rimosus*.

Forward primer (59nt):

5´-**CGA CGA ACG CAT CGC CGC GGC CGC CGC CCG GCT GAC ATG** ATT CCG GGG ATC CGT CGA CC- 3´

Reverse primer (reverse complement, 58nt):

5´-**GGA GGA CGA TGG TTT CTG ATC GCC GCC TCA CCC TCC TCA** TGT AGG CTG GAG CTG CTT C-3´

Reaction/cycle conditions:

Forward primer (100pmol);	1µl
Reverse primer (100pmol);	1µl
Template DNA (~25ng.µl^{-1});	1µl
10x Reaction buffer;	5µl
$MgCl_2$ (25mM);	6µl (3mM final concentration)
dNTPs (1.25mM);	8µl
DMSO;	2.5µl (5% final concentration)
DNA polymerase (3.5U.µl^{-1});	0.5µl
dH_2O;	26µl
Total volume;	**50µl**

1: Hot start;	94^{o}C, 2min
2: Denaturation;	94^{o}C, 45s
3: Annealing;	50^{o}C, 45s
4: Elongation;	72^{o}C, 1min 30s (repeat steps 2-4 x 15)
5: Denaturation;	94^{o}C, 45s
6: Annealing;	55^{o}C, 45s
7: Elongation;	72^{o}C, 1min 30s (repeat steps 6-8 x 15)
8: Final Elongation;	72^{o}C, 5 min

2.3.10 Quantification of nucleic acids

Linear DNA fragments were quantified after separation by agarose gel electrophoresis against serial dilutions (10^{0} – 10^{-2}) of λ*Hin*dIII standard marker. Qualitative comparisons between the intensities of DNA fragments of unknown concentration and the marker of known concentration were thus made.

2.3.11 Hybridization: preparation of DIG-labelled DNA probes

Fragments of DNA were primed at random with digoxygenin (DIG) to act as probes for protocols involving DNA hybridization. DIG-labelled DNA probes were used (DIG-DNA Labelling Kit (Roche), **Section 2.1.6)** in conjunction with Hybond-N+ nitro-cellulose membranes (Amersham Biosciences Ltd.). DIG-labelling reactions were conducted accordingly:

1µg purified DNA in 15µl dH_2O was boiled for 10 minutes and placed immediately on ice for at least 1 minute. The following was then added:
2µl hexanucleotide mix
2µl d-NTP labelling mix (1mmol.l^{-1} dCTP; 1mmol.l^{-1}dGTP; 1mmol.l^{-1} dATP; 0.65mmol.l^{-1} dTTP; 0.35mmol.l^{-1} DIG-dUTP)
1µl Klenow enzyme (1U.μl^{-1})

The reaction was incubated at 37^{o}C overnight and arrested by addition of 2µl 0.2M EDTA, pH 8.0. The probe was boiled for 10 minutes and cooled on ice for 1 minute before hybridization at 42^{o}C.

2.3.12 Hybridization: screening of *S. rimosus* M4018 cosmid library

Reagents:
Denaturing Solution: 0.5M NaOH, 1.5M NaCl
Neutralisation solution: 1.5M NaCl, 1M Tris-HCl, pH7.4
20 x SSC: 3M NaCl, 0.3M tri-sodium citrate (used at 2x concentration)
Proteinase K solution: 2mg.ml^{-1} proteinase K in 2x SSC (prepared fresh)

A cosmid library of *S. rimosus* M4018 (Almuteirie, personal comm.) was constructed and probed with *otcX3* (**Section 4.2**). Each transductant was used to inoculate 1ml L broth supplemented with kanamycin and ampicillin in sterile 96-well plates and incubated at 37^{o}C overnight. 1ml sterile 60% glycerol was then added and mixed by

gentle rotation for 10-15 minutes. The 96-well plates were then stored at -70^{o}C. Eight 96-well plates containing the cosmid library were then probed with *otcX3*.

A Hybond-N+ nitro-cellulose membrane was cut to an appropriate size to accommodate four 96-well plates. Co-ordinated grids were marked on to the membrane for each plate, as well as allowing space for a control box. The membrane was placed between two sheets of 3MM Whatman filter paper soaked in dH_2O, wrapped in foil and sterilised by autoclave. An L agar plate supplemented with kanamaycin was prepared to select for both the cosmid library clones and the positive and negative controls (**Section 4.2)**. Positive controls consisted of *E. coli* JM109 (**Section 2.1.1**) transformed with pET33X3 (**Section 2.1.2**). *E. coli* JM109 transformed with Supercos1 (**Appendix A. 10**) or a cosmid known not to contain *otcX3* were used as negative controls. The sterile membrane was aseptically applied to the surface of the agar taking care to remove any air bubbles with a sterile spreader. The 96-well plates were allowed to thaw at room temperature for at least 30 minutes before inoculating 2µl of each glycerol stock on to the grids on the sterile membrane. The plate was incubated overnight at 37^{o}C.

At room temperature, the membrane containing the colonies was lifted from the agar plate and blotted on a dry sheet of Whatman 3MM filter paper. A fresh sheet of 3MM filter paper was soaked in denaturing solution on to which, the membrane was transferred and incubated for at least 15 minutes. After incubation the membrane was air-dried for 1 minute on a dry sheet of 3MM filter paper and transferred to another sheet of 3MM filter paper soaked in neutralisation solution and incubated for 15 minutes. The membrane was air-dried, transferred on to fresh 3MM filter paper soaked in 2x SSC and incubated for 10 minutes. The membrane was placed between two dry sheets of 3MM filter paper and incubated at 80^{o}C for at least 1 hr.

Before hybridization, the membrane was treated with a minimum volume of proteinase K solution at 37^{o}C for one hour to remove cell debris. A fresh sheet of 3MM filter paper wet with sterile dH_2O was pressed over the membrane (using a

spreader to apply equal pressure). The filter paper was then removed carefully and discarded. This process was repeated until all cell debris was removed.

2.3.13 Hybridization: preparation of Southern blots

Reagents:

***Depurination solution**:* 0.25M HCl

***Denaturing solution**:* 0.5M NaOH, 1.5M NaCl

Alkali transfer solution: 0.25M NaOH, 1.5M NaCl

20x SSC: 3M NaCl, 0.3M tri-sodium citrate

DNA fragments of total DNA samples and any controls were separated overnight by electrophoresis. The gel was stained, photographed (**Section 2.3.6**) and rinsed with dH_2O. The transfer of DNA to a Hybond-N+ nitrocellulose membrane under vacuum pressure using VacuGene™ XL apparatus from Amersham Biosciences Ltd was based on the method described by Southern (1975). A sheet of Whatman 3MM filter paper was cut 1cm (length and breadth) larger than the gel and the membrane cut slightly smaller than the gel (~0.5cm or enough to cover the lanes containing DNA) and placed on the filter paper. They were incubated together in dH_2O for 10 minutes and 20x SSC for a further 10 minutes. A plastic (polythene) "mask" was cut slightly smaller than the membrane (~0.5cm). The filter paper and membrane were transferred on to the porous support screen on the VacuGene™ XL apparatus and the mask placed directly over the membrane. The gel was then placed gently on the mask and The VacuGene™ XL apparatus was sealed, clamped and connected to a pump.

Sufficient depurination solution cover the surface of the gel was added and the vacuum pressure stabilised between 50-55mbar. The gel was then incubated under vacuum for 20 minutes. Depurination solution was decanted off and replaced with denaturing solution for 20 minutes. Denaturing solution was decanted off and replaced with 20x SSC for one hour. The membrane was air-dried on a dry sheet of 3MM filter paper and incubated at 80^oC for at least one hour.

2.3.14 Hybridization and detection of DIG-Labelled probes

Reagents:

a) **Stock solutions**:

20x SSC: 3M NaCl, 0.3M tri-sodium citrate (used at 2x and 0.2x)

10% (w/v) SDS*:* used at 1%

Buffer I: 0.1M Maleic acid, 0.15M NaCl, pH7.5

b) **Experimental solutions**:

Pre-hybridization solution: 12.5ml 20x SSC; 500µl 10% (w/v) Sodium lauroyl sarcosine; 100ml 10% (w/v) SDS; 2.5g blocking reagent; 12ml dH_2O; 25ml deionised formamide

Washing buffer: 0.3% (w/v) Tween-20 in buffer I

2x SSC, 0.1% (w/v) SDS: diluted from stock solution

0.2x SSC / 0.1% (w/v) SDS: diluted from stock solution

Buffer II: 1% (w/v) blocking reagent in buffer I (diluted aseptically from a sterile 10% (w/v) stock solution)

Detection buffer: 0.1M Tris-HCl, 0.1M NaCl, 50mM $MgCl_2$, pH 9.5

A nylon membrane prepared for hybridization (**Section 2.3.12-13**) was rolled in a nylon mesh and incubated in a hybridization chamber, rotating at 42^{o}C for one hour with approximately 25ml pre-hybridization solution, which was decanted off and retained. The DIG-labelled probe was boiled for 10 minutes, incubated on ice for 1 minute then added to the decanted pre-hybridization solution. The membrane was incubated with the probe/pre-hybridization solution, rotating overnight at 42^{o}C. The probe/prehybridization solution was recovered and stored at -20^{o}C.

The membrane was incubated at room temperature for 5 minutes with washing buffer under rotation. It was then washed twice for 15 minutes with 2x SSC/1% (w/v) SDS, and twice at 68^{o}C with 0.2x SSC/1% (w/v) SDS. The membrane was incubated with 40ml buffer II for 30 minutes at room temperature under rotation. Anti-DIG-AP anti-Fab fragments (Roche) were prepared by centrifugation at 13,000g, 10 minutes. 3µl were extracted from the surface and diluted in 20-30 ml fresh buffer II (~1:10,000

dilution). The membrane was incubated with the antibody solution for 30 minutes and subsequently washed twice with washing buffer for 15 minutes and incubated with ~20 ml detection buffer for 5 minutes. The membrane was removed from the hybridization chamber and placed in a sealed polythene bag with 10ml NBT-BCIP in dH_2O (prepared fresh by dissolving a NBT/BCIP tablet (Roche) in 10ml dH_2O). After confirmation of hybridization by production of blue precipitate, the membrane was washed with dH_2O and dried.

2.4 Protein Methods

2.4.1 General reagents for SDS-PAGE

10x SDS-PAGE electrophoresis buffer: 15.1g Tris, 94g glycine per 1L dH_2O. Not sterilised. Diluted for use at 1x concentration with (10% (w/v)) SDS solution added to a final concentration of 1%.

2x SDS sample loading buffer: 125mM Tris pH6.8, 4% (w/v) SDS, 20% (w/v) glycerol and 0.007% (w/v) bromophenol blue. Mercaptoethanol added to 10% (v/v) final concentration immediately before use.

Coomassie Blue SDS-PAGE stain: dH_2O, methanol and glacial acetic acid mixed to a ratio of 6:3:1, respectively. 1g coomassie blue was added per 1L and solubilised overnight in a dark container, stirring. The solution was filtered before use.

SDS-PAGE de-stain: As above without coomassie blue.

Resolving gel buffer: 54g Tris, 6ml 20% (w/v) SDS per 300ml dH_2O, pH 8.8,

Stacking gel buffer: 18g Tris, 6ml 20% (w/v) SDS per 300ml dH_2O, pH 6.8.

Gel-fixing solution: 35% (v/v) ethanol, 2% (v/v) glycerol.

2.4.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE): assembly

SDS-PAGE analysis was used to analyse protein samples. Cell pellets from 1ml samples of *E. coli* cultures over-expressing *otcX3* (**Section 2.2.15**) were resuspended in 100-200µl of 2x SDS sample loading buffer, depending on viscosity. Protein samples collected from column elutions were added to an equal volume of loading buffer.

A 10% (w/v) resolving gel was used to separate and resolve protein samples. A 5% (w/v) stacking gel was used to load the samples. The experimental assembly was composed of the following ingredients:

Component	Resolving gel (10% (w/v))	Stacking gel (5% (w/v))
Resolving gel buffer	5ml	-
Stacking gel buffer	-	1.25ml
30% (w/v) acrylamide/0.8% (w/v) bis	6.65ml	0.83ml
dH_2O	8.1ml	2.92ml
10% (w/v) ammonium persulphate *	150µl	50µl
TEMED*	50µl	15µl

* ammonium persulphate was freshly made and added last to gel mix

Gels were cast between clean glass plates, usually 10 x 7.3cm. The plates were separated using spacers (~0.75mm thick) and fixed into place on a BioRad® SDS-PAGE assembly dock. Resolving gel mixture was cast to fill approximately 3/4 of the plate volume. The remaining space was filled with isopropanol and the gel left to set for 30 minutes. The isopropanol was drained off and the plate washed with dH_2O and dried. The stacking gel mixture was then applied on top of the resolving gel. Wells were created by insertion of comb and left to set for 10-15 minutes. The comb was removed and the plate(s) assembled on to the BioRad electrophoresis apparatus. 1x electrophoresis buffer was added to the tank ensuring that sample wells were completely submerged. Protein samples could then be loaded.

2.4.3 Analysis of protein samples by SDS-PAGE

Protein markers:

BioRad Broad-Range marker (*kDa*): myosin (203); β-galactosidase (115); bovine serum albumin (93); ovalbumin (48.2); carbonic anhydrase (34.7); soybean trypsin inhibitor (28.2); lysozyme (21.1); aprotinin (7.2). Pre-stained.

Promega Broad-Range marker: nine unspecified recombinant proteins of known molecular weights; 225, 150, 100, 75, 50, 35, 25, 15 and 10kDa.

Crude extracts of cell pellets that had been suspended in 2x loading buffer (**Section 2.4.1**) were boiled for 3 minutes, cooled on ice for 2 minutes. Insoluble matter was pelleted by centrifugation at 13,000g, 4^{o}C for 5 minutes. To each gel, 5µl marker and 15µl from a 100µl sample of cell extract was typically loaded into appropriate sample wells. Gels were electrophoresed at 100V for approximately 1 hour. Gels were removed carefully and submerged in coomassie blue stain (**Section 2.4.1**) for at least 2 hours and de-stained until the resolution of protein bands was complete. Gels were incubated with 35% (w/v) ethanol, 2% (w/v) glycerol for at least 30 minutes. Gels were laminated with the EasyBreeze® (Amersham) drying kit.

2.4.4 Lysis of *E. coli* cell pellets

Reagents:

Lysis buffer: 20mM Na_2HPO_4, 0.65mM EDTA, 500mM NaCl, 10% (w/v) glycerol. Protease inhibitors were added immediately before use: Benzamidine or PMSF at a final concentration of 1mM.

A French pressure cell press was used to lyse larger volumes (50 – 1,000ml) of *E. coli* cell suspensions. A cell pellet from a harvested 50ml culture of *E. coli* (**Section 2.2.14**) was resuspended in 5ml lysis buffer. The cell suspension was processed 2-3 times through a mini-cell French press at a pressure of 500 atmospheres. 1ml samples of the lysate were separated into insoluble and soluble fractions by centrifugation at 13,000g, 4^{o}C for 5 minutes. Soluble material remained in the supernatant, which was transferred to a sterile 1.5ml Eppendorf tube. The insoluble fraction was resuspended in 200µl 2x SDS-loading buffer and soluble fractions were added to equal volumes of loading buffer for analysis by SDS-PAGE. For large cell pellets, a 40K pressure cell was used at a pressure of 1000 atmospheres.

2.4.5 His•tag affinity purification using FPLC

Reagents:

All solutions filtered through 0.45μm membrane:

Lysis buffer: See **Section 2.4.4**

Start buffer: 20mM Na_2HPO_4, 500mM NaCl, 10% (v/v) glycerol, pH 7.4.

Elution buffer: 20mM Na_2HPO_4, 500mM NaCl, 500mM imidazole, 10% (v/v) glycerol, pH 7.4.

Strip buffer: 20mM Na_2HPO_4, 500mM NaCl, 10% (v/v) glycerol, 500mM EDTA, pH 7.4.

Charging solution: 0.1M $NiSO_4$.

Protein storage buffer: 50mM Tris, 5mM EDTA, 50% glycerol, pH 7.4.

OtcX3 was over-expressed in *E. coli* with a C-terminal His•tag fusion sequence (**Section 3.2**) to enable His•tag affinity purification. Calibration of a column containing NTA His•bind resin (Amersham) was necessary to ensure compatibility with buffers during purification. The column was washed with three column volumes of filter-sterilised dH_2O, charged with 2.5ml 0.1M $NiSO_4$ and washed with a further three column volumes of dH_2O. The column was calibrated with five column volumes of start buffer followed by five of elution buffer and another five of start buffer. Disposable His•bind columns (Novagen) were used for small-scale purification or to test the potential efficiency of a protocol before attempting large-scale purification with an automated FPLC appliance.

A cell pellet from 1L of *E. coli* harvested after over-expression of *otcX3* was suspended and lysed in no more than 20ml lysis buffer (**Section 2.4.4**). The crude extract was diluted to 100ml with lysis buffer to reduce viscosity and insoluble cellular matter was pelleted by centrifugation at 13,000g, 4^{o}C for 15 minutes. The supernatant was transferred to a sterile tube and residual insoluble matter removed by further centrifugation of the soluble fraction. Insoluble fractions were discarded or stored at -20^{o}C, as required. Soluble fractions were loaded into the sample-loading column of the FPLC appliance and injected onto the His•bind column in accordance

with the purification protocol, controlled by the remote computer. Elution of protein was monitored by UV absorption at 280nm. Elutions were normally collected in 2ml fractions. When necessary, the column was stripped of Ni^{2+} ions with five column volumes of strip buffer, washed with dH_2O and re-calibrated.

The protocol for His•tag affinity purification under denaturing conditions was similar, except for the addition of 6M Guanidine.HCl or 8M urea in all buffers.

2.4.6 Solubilisation and purification of inclusion bodies

The insoluble fraction from a sample of cell lysate was isolated by centrifugation (**Section 2.4.4**). The pellet, containing partially-purified inclusion bodies of OtcX3 was washed twice by suspension in Tris buffer (pH8.0, 50mM) and the wet weight noted. The protocol for solubilisation of the inclusion bodies using the weak detergent, N-lauroylsarcosine (SLS) was based on that described by Rangan and Smith, (1996).

Typically, a wet weight (mg) to solvent volume (ml) ratio of 1:1 was used. The pellet was resuspended by vortexing in 2% (w/v) SLS in 50mM Tris, 1mM EDTA, pH 8.0 in a 250ml Erlenmeyer flask. $CuSO_4$ and cysteine/cystine were added to a final concentration of 1mM to enhance disulphide bond formation. The suspension was incubated at room temperature, rotating at 150rpm for 90 minutes. Samples were taken at appropriate time intervals to monitor the progressive solubilisation of the protein by measuring the UV absorbance at 330nm. After solubilisation was maximised, remaining insoluble matter was pelleted by centrifugation at 10,000g for 10 minutes. The supernatant containing solubilised protein was transferred to a sterile container and the insoluble pellet discarded.

2.4.7 Dialysis of solubilised inclusion bodies

Samples of solubilised inclusion bodies were dialysed to remove SLS and enable protein refolding. Dialysis membranes were prepared by boiling 10-20cm strips in 1mM EDTA, 2mM sodium bicarbonate, pH 8.0 for 10 minutes, washed thoroughly with dH_2O and boiled again in 1mM EDTA, pH 8.0 for 10 minutes. After cooling, membranes were stored at 4^oC, submerged in 1mM EDTA, 2mM sodium bicarbonate, pH 8.0. Before dialysis, membranes were washed with dH_2O. One end was sealed with a dialysis clip before addition of the sample to be dialysed. The other end was sealed with a second clip.

Samples were dialysed in 50mM Tris, pH 8.0 at 4^oC in a volume at least 50x that of the sample. $CuSO_4$ and L-cysteine/cystine were added at 1mM final concentration. The buffer was changed every 3 hours initially during the first 24 hours and left to dialyse overnight. This process was repeated without the addition of $CuSO_4$ and L-cysteine/cystine during the following 24 hours. Partially-folded insoluble aggregates were removed by centrifugation of the sample at 7,000g, 4^oC, 10 minutes and the supernatant transferred back to the membrane. After 48 hours, the dialysed sample was concentrated using 3.5ml micro-concentrators with a 10kDa filter at 7,000g, 4^oC in preparation for further purification by size-exclusion chromatography. 100µl of dialysed samples was added to an equal volume of 2x SDS-loading buffer with 10% 2-mercaptoethanol for analysis by SDS-PAGE.

2.4.8 Separation of proteins by size-exclusion chromatography

Size-exclusion chromatography (Gel filtration) was used to purify protein samples after dialysis (**Section 2.4.7**). Sephadex G-200 (Sigma-Aldrich) porous beads were used to form a gel matrix. Proteins up to 200kDa could be separated by interaction with the porous beads. Smaller proteins interacted more often with the gel matrix and so were eluted (V_e) later than larger proteins. Molecules with a mass greater than 200kDa blue dextran could not interact with the gel matrix and were eluted in the void volume (V_o).

Sephadex G-200 powder was hydrated and used to fill a 30 x 3cm column (Amersham). The powdered beads were suspended in 50mM Tris, 1mM EDTA, 10% (v/v) glycerol, pH 7.5, which was also used as the elution buffer. The mixture was placed in a water bath at 100^{o}C for approximately 1 hour to hydrate and de-gas the gel slurry that was used to fill the column.

To pack the column, the gel slurry was cooled and any excess buffer decanted off. A pump was attached to the base of the column to create a steady packing flow rate and a buffer reservoir was positioned above the column to create a buffer recycling system. The gel slurry was poured into the column using a feeder funnel containing excess gel slurry using a flow rate of 0.07ml.l^{-1} for approximately 2 hours over to equilibrate the packing volume.

After packing, the column was attached to an automated FPLC appliance and calibrated using a protein calibration kit (**Section 2.1.6**) consisting of the following proteins; blue dextran (2,000kDa – provided void volume, V_o), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa). Each protein was solubilised in 50mM Tris, 1mM EDTA, 10% (v/v) glycerol, pH 7.5 and 1ml samples were prepared to recommended concentrations. Samples were injected onto the column individually and processed through the column at a flow rate of 0.07 ml.min^{-1}. The volume of elution for each protein (V_e) was monitored by UV absorption at 280nm and respective V_e/V_o ratios calculated accordingly (**Section 3.4**).

2.5 Characterization of *S. rimosus* M4018 Δ*otcX3::aac(3)IV-oriT* strains: agar-plug bioassays

Strains of *E. coli* or MRSA were cultured, selecting with tetracycline, as required (**Section 2.2.4**) and 1/100 dilutions of each were prepared using sterile L-broth of which 100µl of each was used to inoculate fresh L-agar plates. Agar plugs of each *Streptomyces* strain were then aseptically transferred onto each L-agar plate inoculated with *E. coli* or MRSA, as well as a plug from an L-agar plate

supplemented with tetracycline/OTC as a control. The plates were then incubated overnight at 30^{o}C.

2.6 TLC analysis of metabolites produced by *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutant strains

Six plates of each strain of *S. rimosus* were grown on SFM at 30^{o}C for 4-5 days. Extracts from each set of plates were prepared by multiple extractions with ethyl acetate. Extractions were repeated until pigment intensity decreased significantly, pooled and concentrated by evaporation at 40^{o}C using a rotary evaporator.

Silica gel 60F_{254} plates (Merck) were used to analyse the metabolic extracts by thin layer chromatography (TLC). A starter line approximately 1cm from the base of the silica plate was marked in pencil and marks for each sample noted at equidistant intervals. Concentrated spots of each concentrated extract and OTC control were then applied by capillary action. Chloroform:methanol:water at a ratio of 9:4:0.3 was used as the mobile phase. Enough solvent mixture was added to cover the base of the chromatography tank. The solvent level did not exceed the height of the marked starting point on the TLC plate (between 0.5-0.75cm). The silica plate was carefully placed into the organic solvent. The plate was removed from the tank once the solvent line reached approximately 1cm from the top. The solvent line was marked accordingly before air-drying the plate. The plate was then placed in a sterile petri-dish and submerged in L-agar inoculated with *E. coli* (**Section 4.3.10**). After incubation overnight at 37^{o}C, retention factors (RF) of zones of inhibition could be calculated.

2.7 HPLC analysis of metabolites produced by *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutant strains

Analysis of metabolic extracts (prepared as described in **Section 2.6**) of *Streptomyces* strains by HPLC (**Section 4.3.9**) was conducted using a 150 x 4.6mm C18 column (Burke Analytical, Glasgow, UK; model no. NLS-21273) and evaporated to dryness. A mixture of methanol, acetonitrile and 0.01M oxalic acid was used as the mobile phase at a ratio of 2:3:16, respectively, as described by Oka *et al*. (1987) and filtered through a 0.45µm membrane. Samples were separated through the column at 30^{o}C by incubation with a heater/chiller (Jones Chromatography, Glamorgan, UK; model no. 7955). This was connected to a manual injection loop (20µl volume; Rheodyne, USA), itself connected to an injector (Thermo-Separation Products; model no. P200). The mobile phase was processed through a degasser (Erma, Tokyo, Japan; model no. ERC-3312) connected to the injector. UV spectrum analysis of OTC indicated peak absorbance at 350nm (Oka *et al*., 1987) in the mobile phase. Therefore, a variable wavelength UV-Vis detector (Varian; model no. 9050) was connected to the column and set to 350nm. The detector was connected to an integrator (Spectra-Physics; model no. SP4290).

2ml of the mobile phase was added to each dried extract and gently shaken for 10 minutes. Samples were filtered through a 0.45µm membrane to remove insoluble particles. Serial dilutions of OTC in 0.1M HCl (pH 2.0) at a stock concentration of 10mg.ml^{-1} were prepared to calibrate the UV-Vis detector and the integrator. The flow rate of the mobile phase through the column was set to 1ml.min^{-1} and the pressure was maintained between 2500-2800 PSI. 20µl samples of the standards and filtered extracts were injected onto the column and the retention time (RT) of each peak of UV absorbance at 350nm was printed by the integrator. The serial dilution of OTC that showed the most similar absorbance readings to the extracts was chosen and the range of absorbance was determined empirically by adjusting the attenuation on the integrator and the % transmittance setting on the UV-Vis detector.

Chapter 3:

Cloning, Expression of *otcX3* and Attempted Purification of OtcX3

3.1 Cloning of *otcX3*

3.1.1 Introduction

The aromatic polyketide antibiotic oxytetracycline (OTC), so-called due to its unique 5'–OH group, is produced by the *otc* cluster of *Streptomyces rimosus* as an important secondary polyketide metabolite. A classic Type II polyketide synthase (PKS) complex, the *otc* cluster is a highly evolved and tightly-regulated system. Each of the 23 ORF's within the *otc* cluster produce gene products that play their respective roles in the biosynthesis of OTC, be it in a catalytic or non-catalytic capacity. As discussed in **Section 1.7** most genes in the *otc* cluster have been characterized and their functions determined biochemically with the exception of those described. The evolutionary survival of the *Streptomyces* genus has clearly been influenced by the efficacy of the antimicrobial compounds produced by these species' as an important part of natural environmental competition with other microorganisms.

The biosynthesis of OTC from the initial production of a 19C nonaketamide backbone through to the final tetracyclic structure was described in detail in **Section 1.7**. Information regarding the sequence of the cluster was accumulated gradually (Butler *et al*., 1989; Kim *et al*., 1994) and was successfully completed more recently (Zhang *et al*., 2006). While many genes have been characterized, there are several genes of the *otc* cluster, including *otcX3*, whose functions need to be defined biochemically (**Section 1.7**) to support the bioinformatic evidence that has defined their putative functions. Our understanding of aromatic polyketide biosynthesis will improve by obtaining the data required to biochemically characterize the roles of those genes of the *otc* cluster whose functions have still to be determined. In turn, this will benefit the ultimate goal towards the development of novel therapeutic polyketides.

As explained in **Section 1.7**, the sequence of *otcX3* initially showed homology to other putative polyketide acyltransferases (AT). Early bioinformatic data indicated high similarity to a gene product, DpsD, from the Type II PKS daunomycin cluster

of *S. peucetius* (Kitamura *et al*., 1981; Grimm *et al*., 1994). It was postulated that *dpsD* was present to act as an acyltransferase for the loading of another non-acetate starter unit, propionyl-CoA (Kitamura *et al*., 1981; Grimm *et al*., 1994). In the *otc* cluster of *S. rimosus*, it was hypothesized that an AT could exist for the purpose of loading a different and unique starter unit, malonamyl-CoA, onto the minimal PKS (Thomas and Williams, 1983b, **Section 1.7)**. The presence of a putative acyltransferase had not been found in other Type II PKS clusters that use more regular starter units, leading to this hypothesized role of *otcX3*. Significantly, an increased number of homologous genes/gene products have been sequenced since and the relevant bioinformatic data are discussed below.

The aim of this work was to test the hypothesis described above. The first stage of this process was to synthesise and clone *otcX3* into expression vectors in order to over-express the gene in *E. coli*. Should this be successful, purification of the recombinant protein would allow the construction of an assay to monitor the affinity of the enzyme to bind with a variety of acyl substrates. In turn, this would provide data required to characterize the function of OtcX3. Furthermore, the interaction of recombinant OtcX3 with other proteins involved with the "early" stages of OTC biosynthesis could be investigated. Shortly after the work in this thesis was completed, data were published that provided further insight into the mechanisms of OTC biosynthesis, including the biochemical characterization of several genes whose function had previously been putative and further information about OTC biosynthesis at early (including the incorporation of malonamyl into the polyketide backbone), mid and late stages was revealed (Zhang *et al*., 2006; 2006b; 2007; 2008; Wang *et al*., 2009). The function of *otcX3* was investigated during this work. The outcome of this is discussed in the appropriate context in **Chapter 5**.

3.1.2 BLAST data for *otcX3*

The sequence of *otcX3* was entered into the NCBI BLAST search engine online and a BLASTn (nucleotide:nucleotide) search against the database was processed. The strongest nucleotide identity (with more than 75% query coverage) was found to the putative AT genes of the clusters from *S. galilaeus* for the biosynthesis of the anthracycline anti-tumour polyketides, aklavinone (73%, Chung *et al*., 2002) and its derivative product, aclacinomycin (72%, Raty *et al*., 2000), as well as the orthologue genes of the Type II PKS daunorubicin biosynthetic clusters of *S. peucetius* (69%, Grimm *et al*., 1994), *Streptomyces* C5 (70%, Ye *et al*., 1994) and the cosmomycin (another anti-tumour polyketide) cluster of *S. olindensis* (69%, Garrido *et al*., 2006). This result seemed logical since the chemical structures of these compounds are very similar and the biosyntheses of all commence with the priming of a propionyl-CoA unit. Other strong matches included the putative AT from the fredericamycin cluster from *S. griseus* ATCC 49344 (69%, Wendt-Pienkowski *et al*., 2005) and the putative AT from the R1128 module from *Streptomyces* R1128, (66%, Marti *et al*., 2000).

The amino acid sequence of OtcX3 was entered into the NCBI BLASTp search engine *via* the software program, Geneious©. The strongest matches (Bitscores) to OtcX3 that were derived from Type II systems were used to form the summary displayed in **Table 3.1**. The strongest identity was observed with the putative sister AT's, AknF (i)/(ii), of the aklavinone and aclacinomycin biosynthetic clusters, which had 71, 70% similarity, 63, 62% identity, respectively, to OtcX3. Another AknF homologue from *Streptomyces* SPB74 (Fischbach *et al*., unpublished data) showed a slightly weaker match, with 69% similarity, 60% identity. Another putative AT from the producer of the pristinamycins, *S. pristinaespiralis*, annotated as OxyP (Fischbach *et al*., unpublished data), showed 63% similarity, 50% identity. The putative sister AT's from the daunorubicin-producing clusters of *Streptomyces* C5 and *S. peucetius*, had 67, 68% similarity, 55, 54% identity, respectively. CosF, from the cosmomycin cluster of *S. olindensis* displayed 68% similarity, 59% identity.

Organism	Protein	Bit Score	% Similarity	% Identity	% Gaps
S. galilaeus	AknF (1)	261.922	71	63	0
S. galilaeus	AknF (2)	250.751	70	62	0
S. C5	OrfD	231.106	67	55	1
S. peucetius	DpsD	230.72	68	54	2
S. olindensis	CosF	228.409	67	58	1
S. griseus	Fdm - ORF2	226.483	65	52	0
S. SPB74	AknF	221.476	69	60	0
S. pristinaespiralis	OxyP	212.616	63	50	0
Salinispora tropica CNB-440	Acyltransferase region – FAS	196.052	61	49	1
S. griseoruber	Hed - ORF8	191.815	57	46	0
S. R1128	ZhuC	184.882	61	49	2
S. maritimus	EncL	182.956	56	43	0
T. fusca YX	Put. AT Modular PKS	172.17	58	45	1

Table 3.1 Summary of relevant peptide sequence BLAST data of proteins displaying the highest similarity to OtcX3 from current database

The R1128 PKS from the organism, *Streptomyces* R1128, has a flexible initiation module, which produces one of four R1128 compounds (A-D) depending on the starter unit incorporated (Hori *et al*., 1993a; 1993b). This initiation module accepts an acetoacetyl, β-ketopentanoyl, 3-oxo-4-methylpentanoyl, or β-ketohexanoyl starter unit and has a protein, ZhuC, which displays 61% similarity, 49% identity with OtcX3 (Marti *et al*., 2000). The enterocin PKS from the marine strain, *S. maritimus*, uses another unique starter unit, benzoyl-CoA (Piel *et al*., 2000). The protein EncL, from this PKS showed 56% similarity, 43% identity. A homologue of OtcX3 (Fdm-ORF2) was also found from the PKS for the anti-tumour drug, fredericamycin from *S. griseus* that displayed 65% similarity, 52% identity. This is one of the few instances from the BLASTp data that shows similarity to a gene from a pathway that does not use a unique starter unit, as the polyketide backbone of fredericamycin is built from the condensation of 15 malonyl units (Wendt-Pienkowski *et al*., 2005).

The PKS from *S. griseoruber* for the anti-tumour drug, hedamycin is unusual in that a Type I PKS is used to synthesise a unique starter unit, hexanoate, which is subsequently used to prime a Type II PKS phase (Bililign *et al*., 2004). One protein from this cluster, Hed-ORF8, displayed 57% similarity and 46% identity to OtcX3. Only 2 of the top 14 matches were from non-streptomycete origins: one putative AT, from the actinomycete *Thermobifida fusca* YX was the weakest match with 58% similarity, 45% identity (Lykidis *et al*., 2007); the other, also from an actinomycete, *Salinispora tropica* (Coleman *et al*., unpublished data), predicted to function as part of a Type II FAS, showed 61% similarity and 49% identity.

The data from these matches were formed into a Global sequence alignment to identify conserved amino acid sequences using the program ClustalW from www.ebi.ac.uk, using the Blosum cost matrix (Henikoff and Henikoff, 1992) with a 'gap open cost' value of 10 and a 'gap extend cost' value of 0.1. This was processed *via* the software program Geneious©. The sequence alignment is illustrated in **Figs 3.1(i)** and **(ii)**. Most of the highly conserved regions/motifs identified in the consensus sequence were dominated by more internal hydrophobic amino acids with conserved hydrophilic regions occurring less frequently that are likely to represent

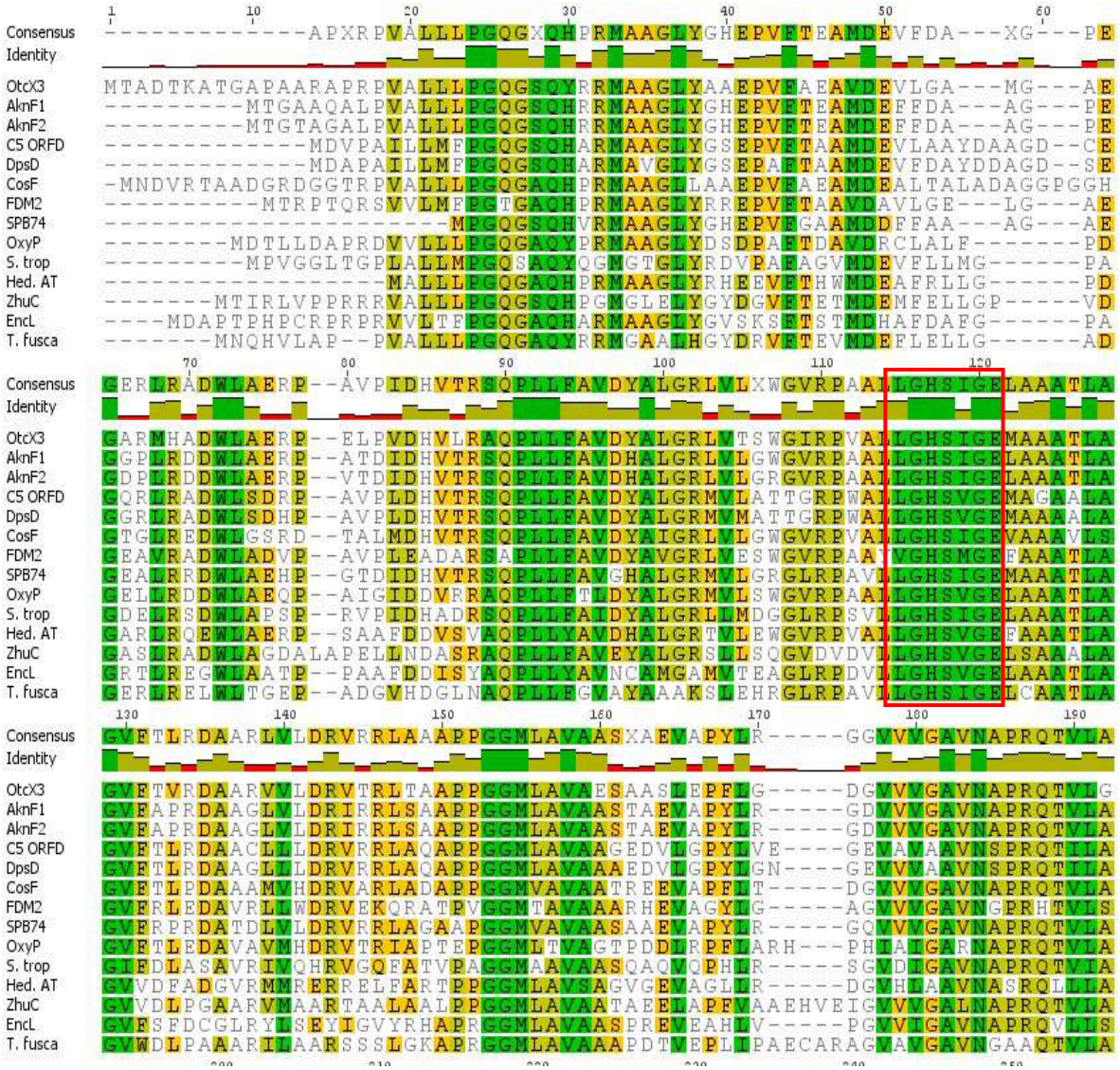


Fig 3.1(i) Part A of Global Sequence Alignment of BLASTp data for OtcX3

Residues highlighted in **green** – 80-100% conservation

" " **brown** – 60-80% conservation

" " **orange** – 40-60% conservation

Motif highlighted by red box indicates conserved active site containing serine residue

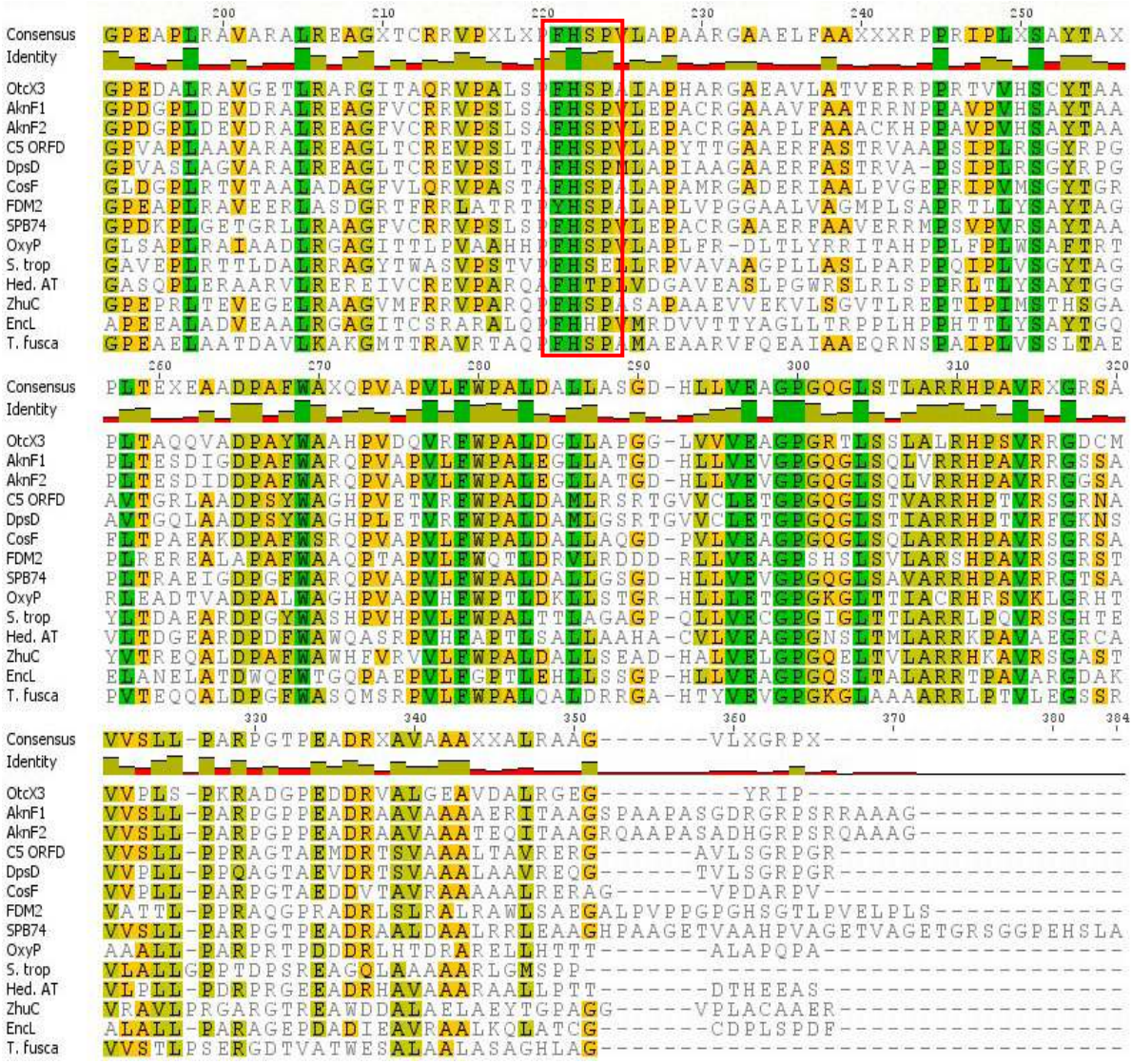


Fig 3.1(ii) Part B of Global Sequence Alignment from BLASTp data

Residues highlighted in **green** – 80-100% conservation

" " " **brown** – 60-80% conservation

" " " **orange** – 40-60% conservation

Conserved motif highlighted by red box indicates conserved active site containing histidine residue.

important globular surface residues. More significantly, the sequence alignment revealed two highly conserved motifs believed to hold important residues for active sites with acyltransferase function according to the Pfam database (Finn *et al*., 2008): the first highly conserved motif had the consensus identity of L-G-H-S-I-G-E and was recognised as having an active site serine residue; the second, F-H-S-P was recognised as having an active site histidine residue. In certain cases the isoleucine of the first catalytic motif was replaced by a valine or methionine residue. The presence of these hydrophobic amino acids surrounding the hydrophilic serine and histidine residues are most likely needed to maintain important structural integrity around the active sites to ensure the affinity required to bind a specific acyl substrate.

The sequence alignment in **Fig 3.1** highlighted the conserved regions common to each of the proteins. This alignment was then used to form a phylogenetic tree to gain a more diagrammatical representation of the relationship between OtcX3 and some of the proteins in the alignment. Again, using the software program, Geneious©, the global alignment data of the top fifty BLAST matches to OtcX3 was processed through the PHYML software program using the WAG amino acid substitution model (Guindon *et al*., 2005; Whelan and Goldman, 2001) that produces a 'maximum likelihood' tree based on the dataset processed. The phylogenetic tree was produced and displayed in **Fig 3.2**. The branch lengths were a measure of the amount of divergence between two nodes in the tree. The unit of measure for the branches and scale bar represented the number of amino acid substitutions per site. The protein with the lowest BitScore from the BLASTp search, a β-ketosynthase from the organism *H. aurantiacus* (located at the top of the tree) was used as the out-group.

From the tree generated by the top fifty BLASTp matches to OtcX3, the putative homologous AT's from Type II PKS clusters clearly formed an exclusive clade as illustrated in **Fig 3.2**. This reinforced the evidence from the literature that OtcX3 and its closest known relatives may have similar roles in their respective PKS complexes. The same dataset was bootstrapped 8 times. In each instance slight variations amongst the fifty proteins could be observed but the Type II proteins were always

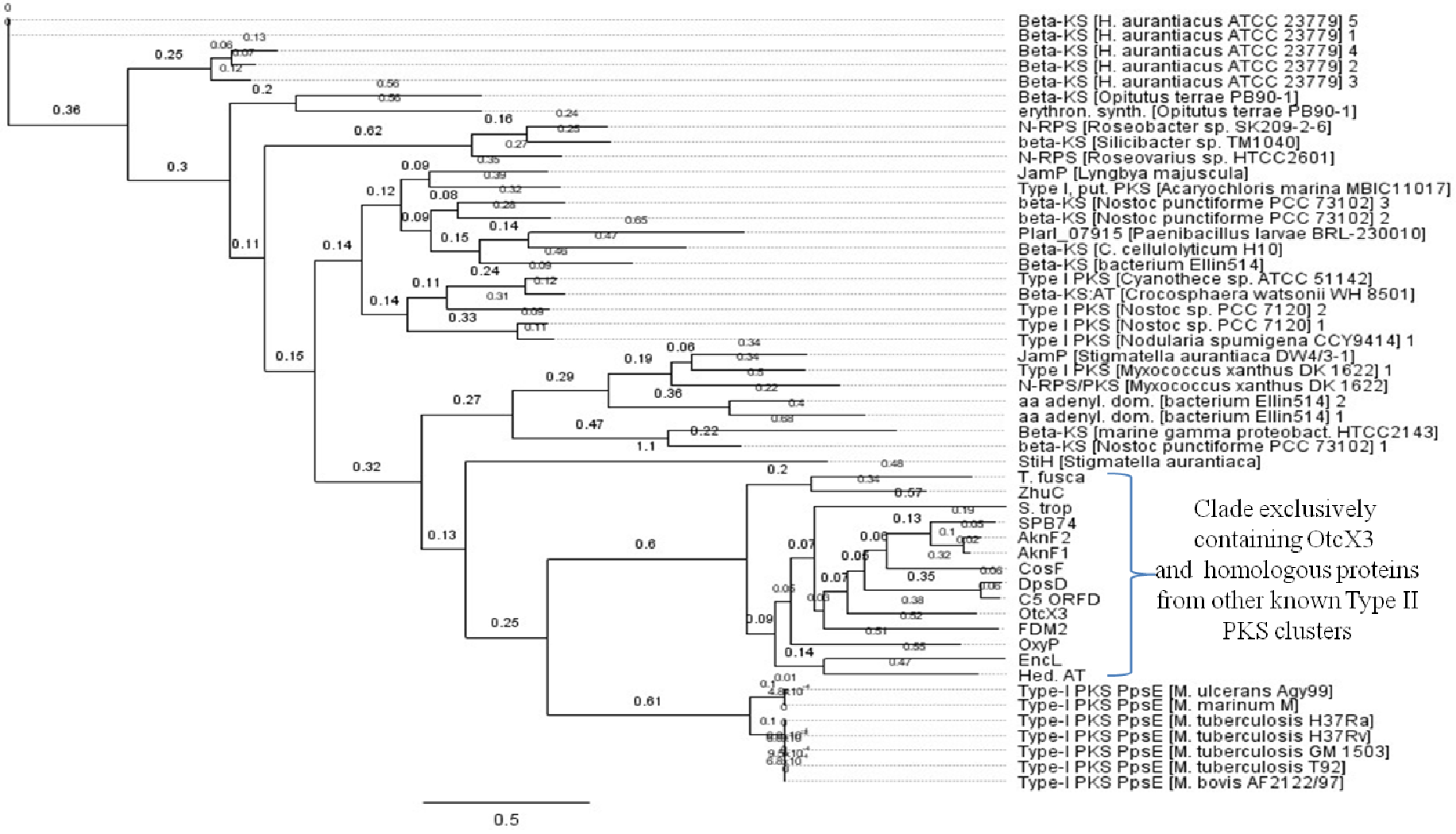


Fig 3.2 Phylogenetic tree outlining evolutionary relationships between OtcX3 and the proteins of known sequence in the BLASTp database

clustered into a single clade with very similar topologies in each instance. The tree illustrated represented the most commonly observed topology. The reason for using such a large dataset was that only thirteen homologous proteins from Type II clusters homologous proteins to OtcX3 were found, none of which had been biochemically characterized, with the exception of ZhuC (see **Chapter 5** for further discussion of ZhuC in relevance to OtcX3). Therefore, by including other proteins from non-Type II backgrounds (Type I modular proteins, β-ketosynthases etc.), a clearer picture of the relationship between these proteins might be observed.

The clade illustrated in **Fig 3.2** is divided. In the first sister clade, the data indicate that the putative AT from *T. fusca* YX and ZhuC from *Streptomyces* R1128 evolved separately. Therefore, these two proteins might be subtly different in function from their other Type II homologues. The other sister clade shows that AknF1 and its sister taxon, AknF2, (both from *S. galilaeus*) are the most evolved proteins in the group, along with the putative AT from *Streptomyces* SPB74 - a closely related sister taxon. The tree also indicated that these proteins were derived from CosF, itself derived from the duo of DpsD and ORFD from *S. peucetius* and *Streptomyces* C5. Since these proteins all come from PKS clusters that incorporate propionyl-CoA as the starter unit, this makes teleological sense for them to be close relatives.

OtcX3 was located on its own branch with no associated sister taxon, consistent with the view that no other known PKS cluster uses malonamyl-CoA as the starter unit. This sub-clade is completed by the putative AT from the fredericamycin cluster; the only cluster from this group known not to incorporate a ‘unique’ priming unit. EncL and the putative AT from the hedamycin cluster (Hed AT) are paired as sister taxa in this tree on an independent branch. Again, this makes teleological sense, since each respective cluster involves incorporation of two very complex starter units: benzoyl-CoA (EncL) and hexanoate (for the Hed AT).

3.1.3 Synthesis of *otcX3* by PCR

otcX3 was synthesised by PCR using Red Taq polymerase, as described in **Section 2.3.8.** The template plasmid, pSRG3 (Garven, 1995) contained a contiguous 5.2kb *Pst*I fragment of the *otc* cluster including the complete *otcX* locus, *otcC* and segments of the *otcD5* and *otcZ* ORF's. Primers were designed to incorporate the restriction sites *Nde*I and *Bam*HI into the PCR product for cloning purposes. *OtcX3* was amplified successfully (**Fig 3.3**) at a size consistent with the *in silico* analysis. The PCR product was purified from a 1xTAE agarose gel, eluted in filtered, sterile dH_2O and sent for sequencing.

3.1.4 Cloning of *otcX3* PCR product

The PCR product (**Section 3.1.3**) was subsequently treated with an end-conversion mix (Novagen) to remove 'sticky ends' from the PCR product and ligated into the pT7Blue3 vector (Novagen). Sequence analysis of plasmid DNA isolated from an insert-containing transformant revealed a silent mutation indicated by the change of a GGC codon to GGT, both of which encode glycine (**Fig 3.4).**

The Novagen expression vector, pET15b, was initially chosen for expression studies. The 1.026kb *Nde*I/*Bam*HI insert was excised from pT7Blue3/*otcX3* and ligated into the polylinker region of pET15b opened with *Nde*I/*Bam*HI, to form the construct pET15X3. After confirmation using *Nde*I and *Bam*HI, the construct was introduced into *E. coli* expression strains: BL21::DE3; BL21/pLysS::DE3; HMS174::DE3; Rosetta::DE3 and Tuner placI::DE3. The ligation of *otcX3* was repeated using the similar vector pET33b(+) to form pET33X3 (see **Fig 3.5** for restriction analysis) following problems encountered with over-expression of the gene with pET15b (discussed in **Section 3.2).**

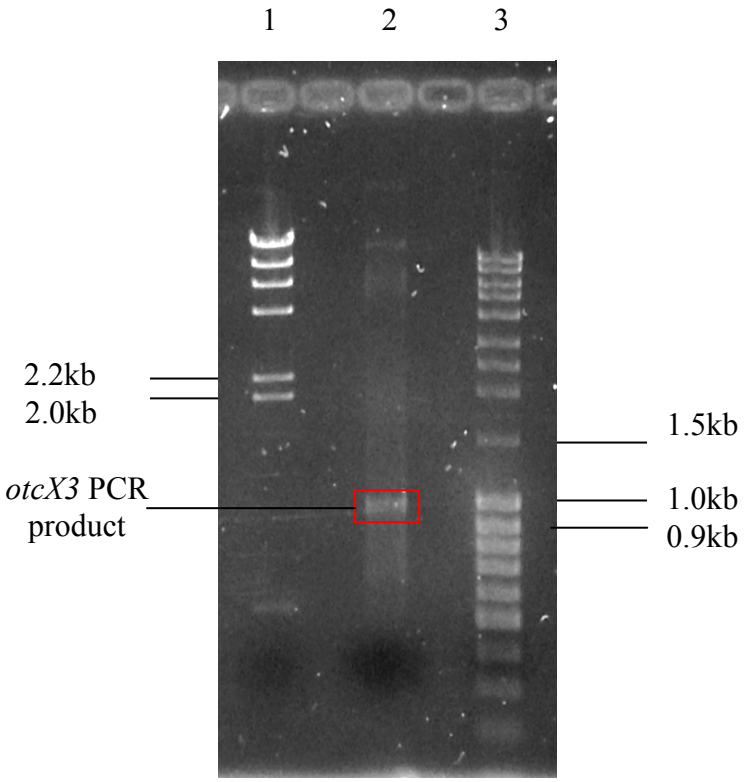


Fig 3.3 amplification of *otcX3* by Red Taq Polymerase

Lane 1: 250ng λ *Hin*dIII marker (Promega)

Lane 2: 5ng *otcX3* PCR product

Lane 3: 1Kb Promega DNA marker

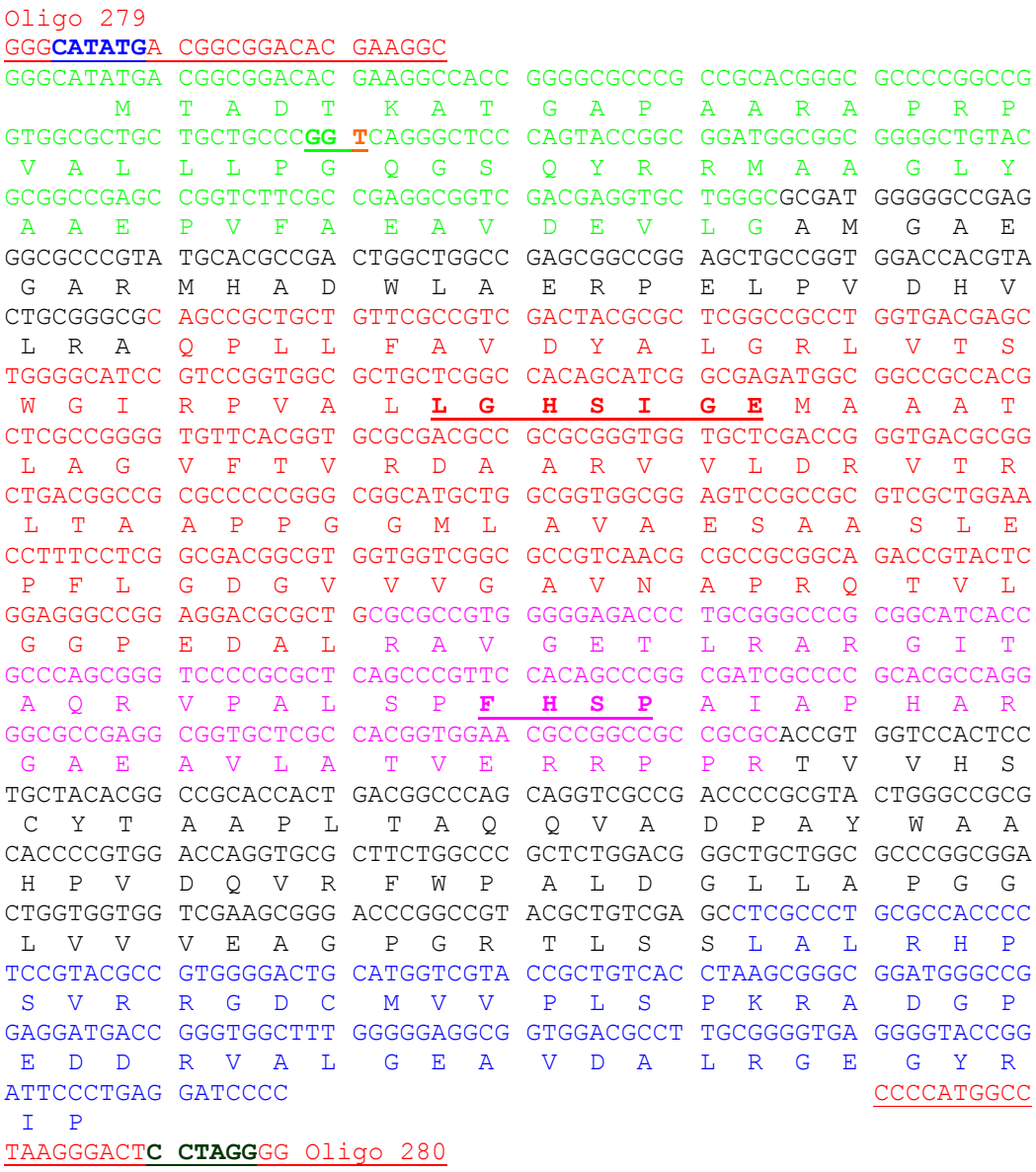


Fig 3.4 *otcX3* PCR product sequence (including primers)

*Nde*I and *Bam*HI sites highlighted in bold **blue** and **dark green**, respectively. The silent mutation of a cytosine to a thymine nucleotide is highlighted in bold **orange**. Highlighted nucleotide/peptide sequences correspond to the sequence data in **Appendices A. 1 – A. 4**; highlighted in red is the sequence data from **Appendix A. 1**, sequences in pink from **Appendix A. 2**, sequences in green from **Appendix A. 3** and the sequences in blue from **Appendix A. 4**. Peptide sequences indicated no changes to the amino acid sequence were observed after translation of the sequences. Putative acyltransferase domains indicated by underlined residues.

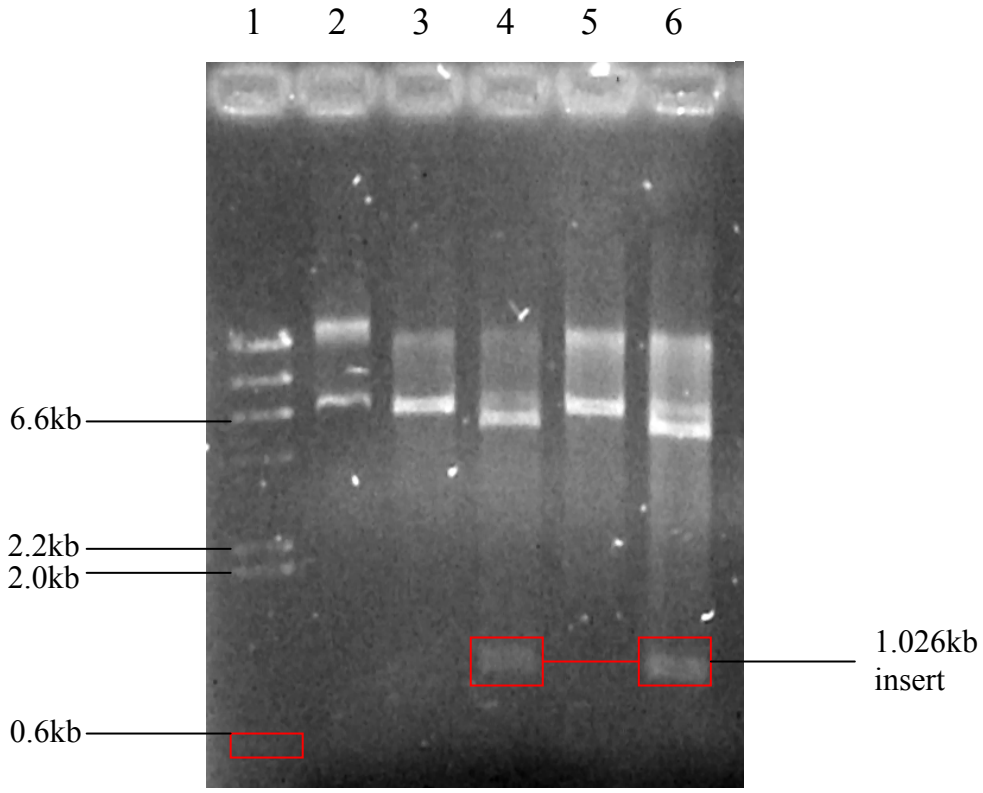


Fig 3.5 pET33X3 isolated from *E. coli* DS941 cut with *Nde*I and *Bam*HI

Lane 1: 250ng λ *Hin*dIII Promega DNA marker

Lane 2: 100ng uncut pET33X3 as digested in lanes 3 and 4

Lane 3: 100ng clone containing 6.4kb pET33X3 linearised by *Nde* I

Lane 4: 100ng clone containing 6.4kb pET33X3 digested with *Nde*I/*Bam*HI, cleaving 1.026kb insert from 5.4kb vector

Lanes 5 and 6: as 3 and 4 except different DNA preparation from separate transformant

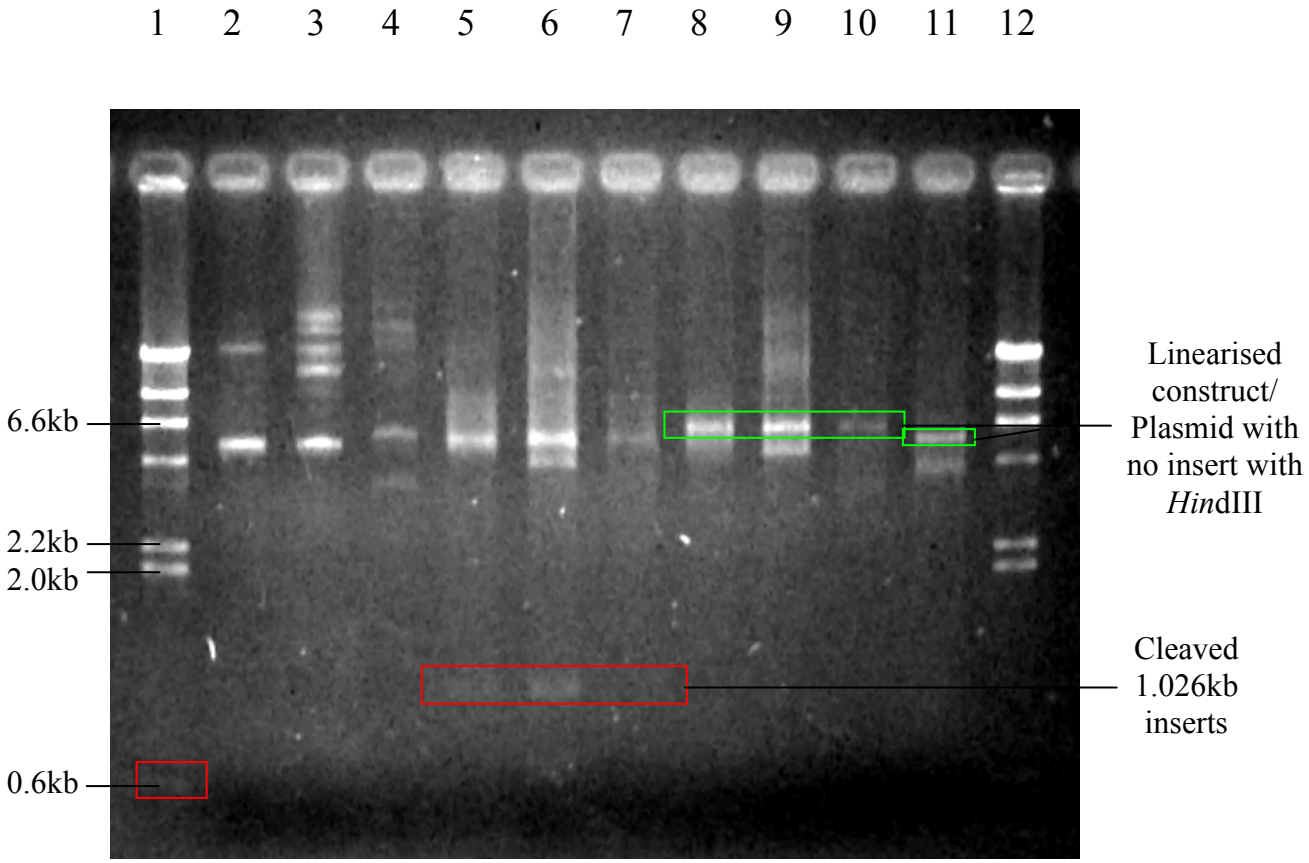


Fig 3.6 Digestion of pET33X3 isolated from *E. coli* expression strains with *Nde*I/*Bam*HI

Lane 1/12: 250ng λ *Hin*dIII Promega DNA marker

Lanes 2-4: 40, 50 and 10ng uncut DNA from HMS174::DE3, BL21::DE3 and Rosetta::DE3 strains, respectively

Lanes 5-7: 40, 50 and 10ng pET33X3 isolated from HMS174::DE3, BL21::DE3 and Rosetta::DE3 strains, respectively digested with *Nde*I/*Bam*HI to cleave 1.026kb insert (insert not visible in lane 7 due to weak concentration of DNA)

Lanes 8-10: 40, 50 and 10ng pET33X3 isolated from HMS174::DE3, BL21::DE3 and Rosetta::DE3 strains, respectively linearised to 6.4kb by digestion with *Hin*dIII

Lane 11: 15ng pET33b(+) vector (no insert) linearised by *Hin*dIII to 5.4kb.

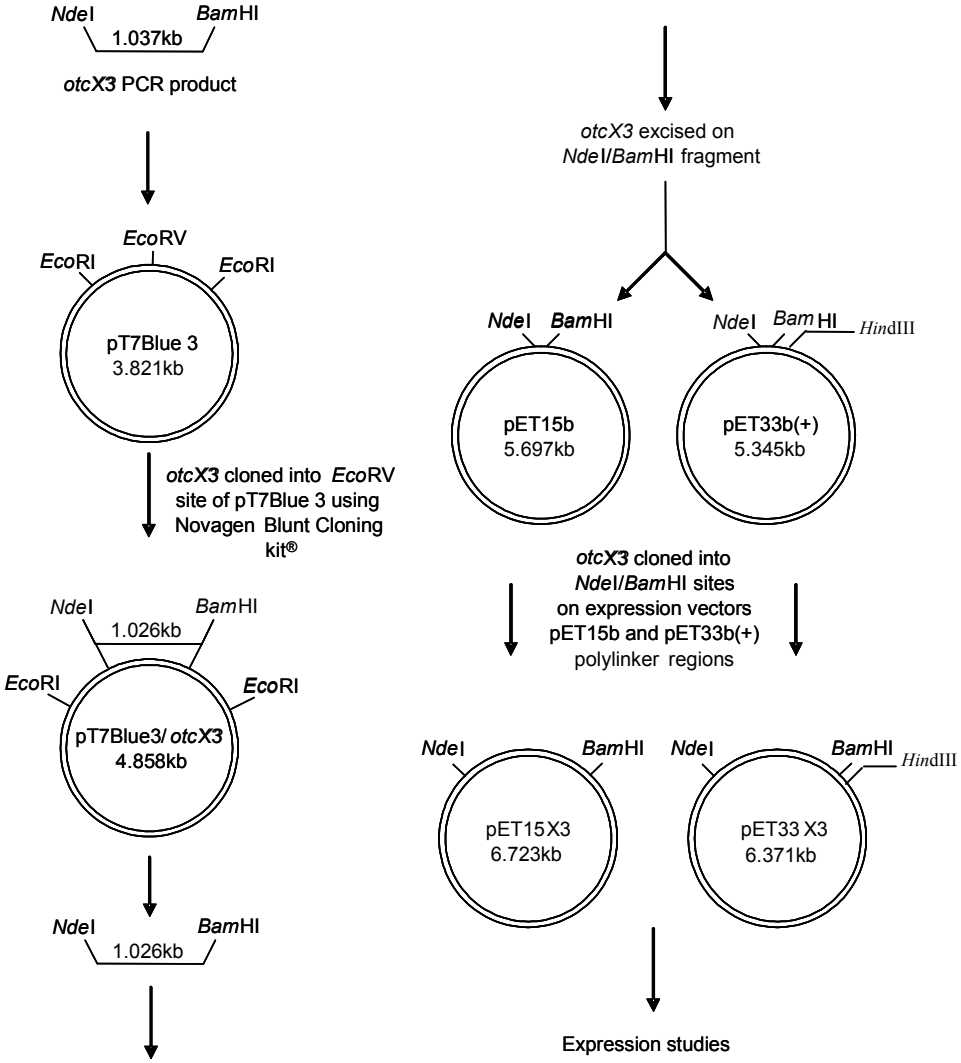


Fig 3.7 Flow chart illustrating cloning procedure

For plasmid maps, see **Appendix A. 5** and **A. 6.**

3.2 Over-expression of *otcX3* in *E. coli*

The high G+C content of *Streptomyces* DNA can lead to problems when attempting to express a *Streptomyces* gene using *E. coli* as a host. For example, some of the codons in *Streptomyces* DNA may have too low abundance of complementary tRNA anti-codons in the cytoplasm of *E. coli* to translate the mRNA sequence efficiently. Examples of codons that are rarely used by *E. coli* (Chen and Inouye, 1990) include:

Arginine – AGA, AGG, CGG, CGA
Isoleucine – AUA
Leucine – CUA
Glycine – GGA
Proline – CCC

Novagen have developed a wide range of *E. coli* expression strains based on the *E. coli* B and K12 strains. Of those available in the laboratory at the time of study, the only strain derived from K12 was HMS174. Those derived from the B strain included BL21, BL21/plysS, Origami™ B, Rosetta™ and Tuner™ strains (**Section 2.1.1)**. These strains lack the *lon* protease and *ompT* outer membrane protease, which reduces the chance of proteolytic degradation of the recombinant protein after cell lysis (Grodberg and Dunn, 1988). Strains containing the plysS plasmid produce a small amount of T7 lysozyme that is non-lethal to the cell, since the lysozyme cannot penetrate the inner membrane, thus preventing peptidoglycan degradation in the cell wall and eventual cell lysis (Inouye *et al*., 1973). T7 lysozyme binds to and inhibits T7 RNA polymerase, thus repressing the rate of expression (Moffat and Studier, 1987; Studier 1991) and encouraging appropriate protein folding. This can also be a useful approach with a construct that is expressing a toxic recombinant protein.

Derived from the BL21 strain, the Tuner™, Origami™ and Rosetta™ strains are *lacYI* (lac permease) mutants allowing adjustable levels of IPTG induction. The level of protein solubility can be modulated by slowing the rate of expression and reducing the aggregation of inclusion bodies in the cytoplasm. Origami™ B also has *trxB*

(thioredoxin reductase) and *gor* (glutathione reductase) mutations that encourage correct disulphide bond formation in the cytoplasm. The Rosetta™ strain carries a plasmid encoding chloramphenicol resistance, pRARE (derived from pRIG, Baca and Hol, 2000) that encodes tRNAs for codons that *E. coli* rarely uses, listed above, thus providing a potential solution to achieving enhanced or successful expression (Brinkmann *et al*., 1989; Seidel *et al*., 1992; Rosenberg *et al*., 1993; Del Tito *et al*., 1995).

pET15b and pET33b(+) are similar vectors, based on the plasmid pBR322 (Sutcliffe, 1979), but differ in a few characteristics (see **Appendices A. 5** and **A. 6**). Both vectors can express recombinant proteins with His•tag fusions using the T7*lac* promoter. pET15b encodes a single N-terminus 6-residue His•tag, while pET33b(+) provides dual 6-residue His•tags at both the C- and N- termini although the presence of a stop codon in the *otcX3* insert prevented transcription of the C-terminus His•tag. This makes both vectors ideal for purification using His•bind resin, although the His•tag fusion sequences increase the chances of interfering with the natural folding patterns of the protein of interest.

For reasons that were unclear, it was impossible to detect any expression of *otcX3* using pET15b in any of the strains described. The use of alternative media e.g. terrific broth or the alteration of growth temperatures ranging from 18-37^{o}C showed no benefits. Rosetta™::DE3, containing the pRARE plasmid, also showed no expression of *otcX3*, suggesting that rare codon usage was not the issue.

Over-expression from pET33X3 (**Section 2.2.15**) was observed in each strain (except for the incompatible Origami B::DE3 strain) by SDS-PAGE analysis (**Section 2.4.3**) and, for each, the recombinant protein tracked near the 35 kDa standard marker, as predicted for OtcX3. Considering the similarity between pET15b and pET33b(+), this result indicated that there must have been a problem with the pET15X3 construct that prevented transcription of *otcX3* mRNA. Expression was achieved initially with the HMS174::DE3 strain using standard L broth growth media and IPTG induction at a final concentration of 1mM. Expression levels were also found to vary between

each strain used. Subsequently, a range of growth temperatures (18, 30 and 37^{o}C) and IPTG induction concentrations (0.1-1mM – on lac permease mutant strains only) were studied in an attempt to optimize conditions for over-expression.

Further analysis at 18^{o}C and 30^{o}C showed that induction at 30^{o}C using a final concentration of 1mM IPTG gave the clearest and highest expression levels, e.g. Lanes 2-8 in **Fig 3.8).** Over-expression peaked between 2 and 3 hours before decreasing after 3 hours. This indicated that the recombinant protein was either unstable in and/or toxic to the host cell. After 8 hours, expression levels were lower than observed after 1 hour and only a trace of recombinant protein could be seen in the overnight sample.

E. coli strains BL21::DE3, BL21/plysS::DE3 and Rosetta™::DE3 all showed lower expression levels compared to HMS174::DE3. However, the Tuner™ strain showed the highest expression levels with growth conditions at 30^{o}C in standard media and IPTG induction at 1mM (**Fig 3.9**). Degradation of the protein after approximately 3 hours was also observed in Tuner™::DE3. The low level of expression in Rosetta™::DE3, essentially the same strain as Tuner™::DE3 but with the inclusion of the pRARE plasmid, was unexpected. This also confirmed that the additional tRNAs supplied on the pRARE plasmid did not provide any assistance to *E. coli* in translation of *otcX3*.

50ml cultures of *E. coli* HMS174::DE3 and Tuner::DE3 expressing *otcX3* were harvested and lysed (**Section 2.4.4)**. Final IPTG induction concentrations of 0.25, 0.5 and 1mM were tested with the Tuner::DE3 strain; however, there did not appear to be any noticeable variance in expression levels at lower IPTG concentrations. After lysis by French Press, insoluble and soluble fractions were separated by centrifugation and analysed by SDS-PAGE. **Figs 3.11** and **3.12** illustrated the soluble and insoluble fractions from expression in *E. coli* HMS174::DE3 and Tuner™::DE3, respectively. It was clear that in each strain, OtcX3 was expressed mainly as inclusion bodies with only minimal evidence of recombinant protein in the soluble fraction.

The formation of inclusion bodies in each strain tested indicated that the over-expressed protein could not fold correctly in *E. coli*. There were many possible reasons why this may have occurred. However, they remain purely speculative. These included: possible folding interference caused by the His•tag fusion; deficiency of *E. coli* to correctly modify the protein after translation; the transcription rate of *otcX3* induced by IPTG was too strong to enable correct folding; the intracellular environment of *E. coli* did not allow for the formation of OtcX3 as a soluble protein.

Attempts were made to enhance the formation of soluble OtcX3 by changing the growth medium and using terrific broth and lowering the growth temperature to 18^{o}C. Lower expression levels were observed at 18^{o}C but the protein was still expressed as inclusion bodies in each strain. Likewise, changing the growth medium had no effect on the solubility of the over-expressed protein.

The reason for the intracellular degradation of OtcX3 after 3 hours could not be explained. The action of Lon protease in the HMS174::DE3 strain was considered. Lon protease degrades proteins that cannot be folded correctly by *E. coli* chaperones and/or are toxic to the host (Gottesman and Maurizi, 1992; Wickner *et al*., 1999). However, the same degradation after 3 hours was observed in all strains of *E. coli*, all of which were Lon protease-deficient mutants, thus eliminating this as a possible explanation. Therefore, the only conclusion that could be made was that OtcX3 over-expressed from pET33X3 was degraded after 3 hours in each strain of *E. coli* for reasons that were unclear.

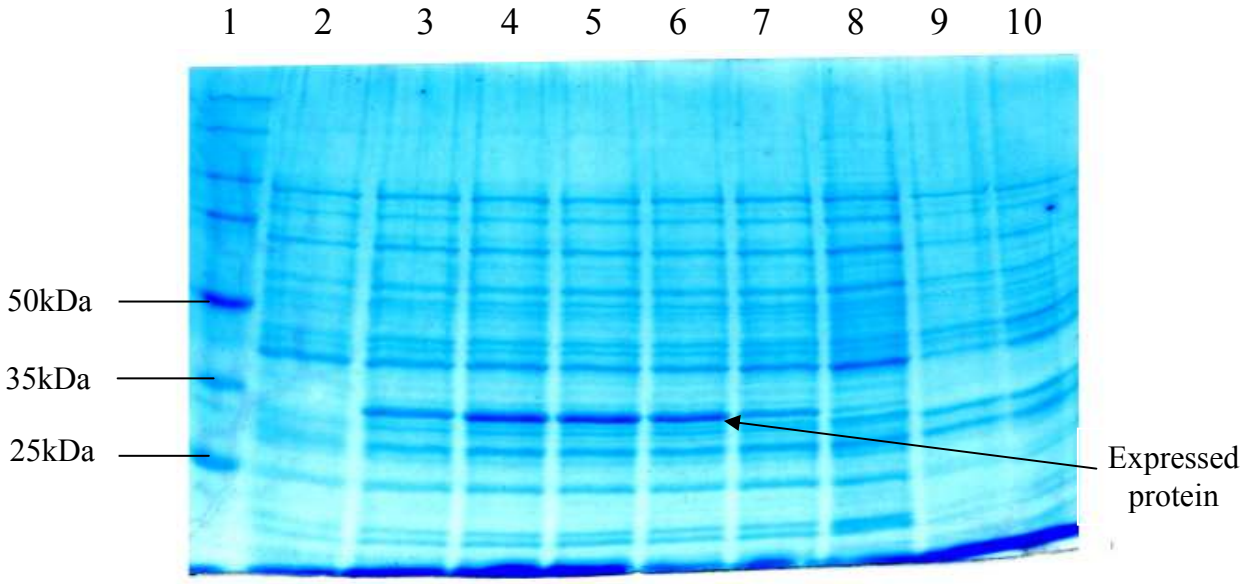


Fig 3.8 SDS-PAGE analysis illustrating expression of *otcX3* in *E. coli* HMS174::DE3 by 1mM IPTG induction in pET33b(+) at 30°C

Lane 1: 5µl Promega broad range protein marker

Lane 2: 15µl sample before IPTG induction (0Hr)

Lane 3: 15µl sample after 1hr IPTG induction

Lane 4: 15µl sample after 2hrs

Lane 5: 15µl sample after 3hrs

Lane 6: 15µl sample after 4hrs

Lane 7: 15µl sample after 8hrs

Lane 8: 15µl of overnight sample

Lane 9: 15µl sample uninduced pET33X3 after 2hrs (control)

Lane 10: 15µl sample induced pET33b vector after 2hrs (control)

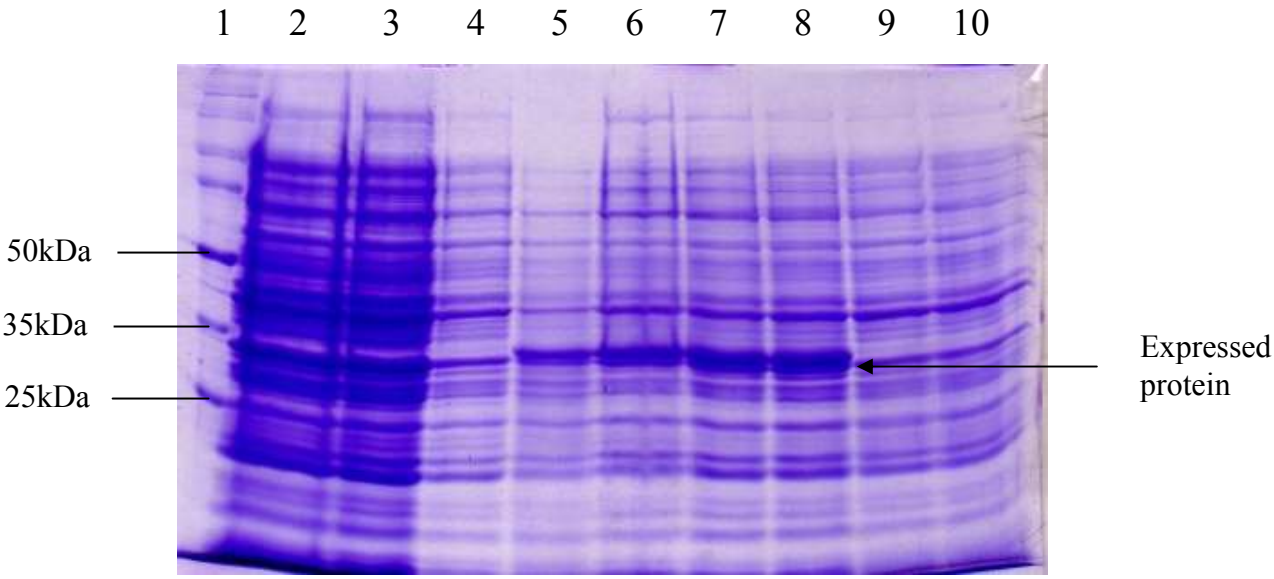


Fig 3.9 SDS-PAGE analysis illustrating expression of *otcX3* in *E. coli* Tuner™::DE3 by 1mM IPTG induction in pET33b(+) at 30°C

Lane 1: 5µl Promega broad range protein marker

Lane 2: 15µl sample uninduced culture 2 hrs after IPTG induction

Lane 3: 15µl sample uninduced culture 3 hrs after IPTG induction

Lane 4: 15µl induced sample at 0hrs IPTG induction

Lane 5: 15µl induced sample after 1hr

Lane 6: 15µl induced sample after 2hrs

Lane 7: 15µl induced sample after 2.5hrs

Lane 8: 15µl induced sample after 3hrs induction

Lane 9: 15µl sample induced pET33b negative control after 1hr

Lane 10: 15µl sample induced pET33b vector after 2hr

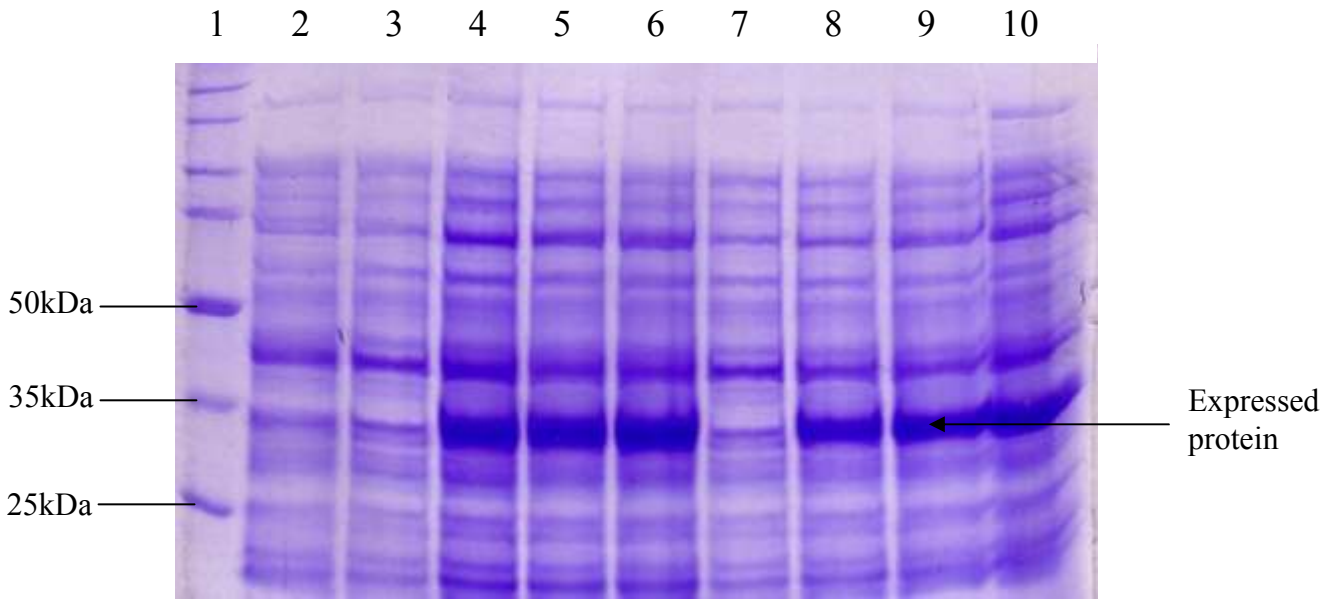


Fig 3.10 SDS-PAGE analysis illustrating expression of *otcX3* in Tuner™::DE3 by 0.5 and 0.25mM IPTG induction in pET33b(+) at 30°C

Lane 1: 5µl Promega broad range protein marker

lane 2: 15µl sample uninduced culture 1hr after IPTG induction

lane 3: 15µl sample induced culture at 0hrs 0.25mM IPTG induction

lane 4: 15µl induced sample after 1hr 0.25mM IPTG induction

lane 5: 15µl induced sample after 2hrs 0.25mM IPTG induction

lane 6: 15µl induced sample after 2.5hrs 0.25mM IPTG induction

lane 7: 15µl sample induced culture at 0hrs 0.5mM IPTG induction

lane 8: 15µl sample induced culture at 1hr 0.5mM IPTG induction

lane 9: 15µl sample induced culture at 2hrs 0.5mM IPTG induction

lane 10: 15µl sample induced culture at 2.5hrs 0.5mM IPTG induction

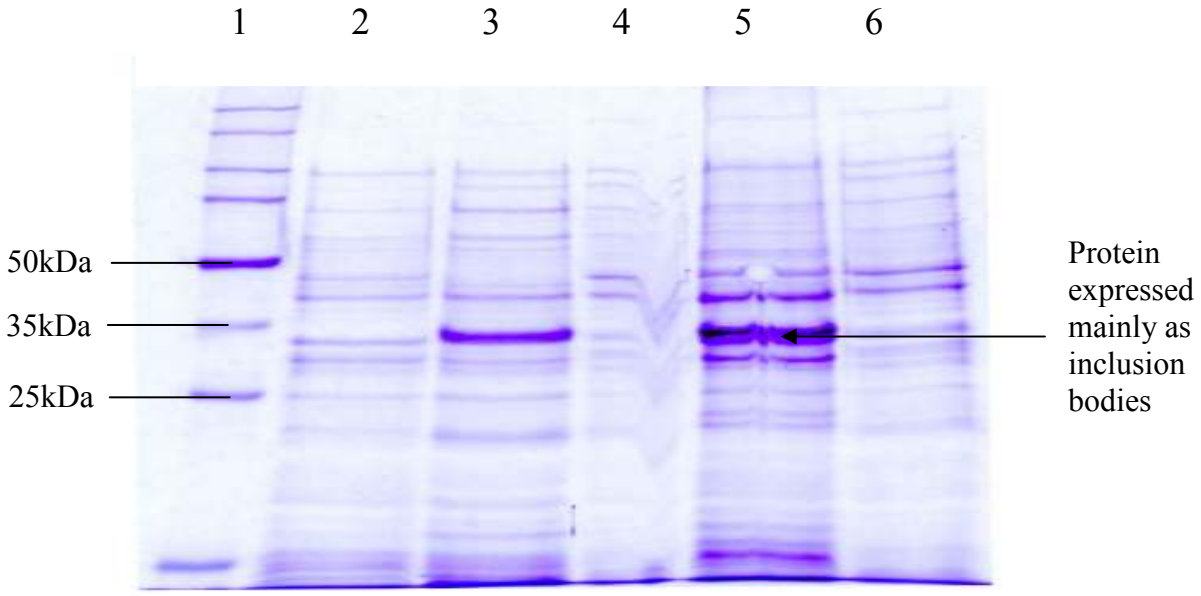


Fig 3.11 SDS-PAGE analysis illustrating formation of OtcX3 as inclusion bodies in *E. coli* HMS174::DE3

Lane 1: 5µl Promega broad range protein marker

Lane 2: 15µl sample induced pET33b(+) vector control after 2hrs

Lane 3: 15µl sample expression of *otcX3* in HMS174::DE3 after 2hrs

Lane 4: 15µl soluble fraction sample after cell lysis by French Press

Lane 5: 15µl insoluble fraction sample after cell lysis by French Press

Lane 6: as lane 4 except with 30µl sample

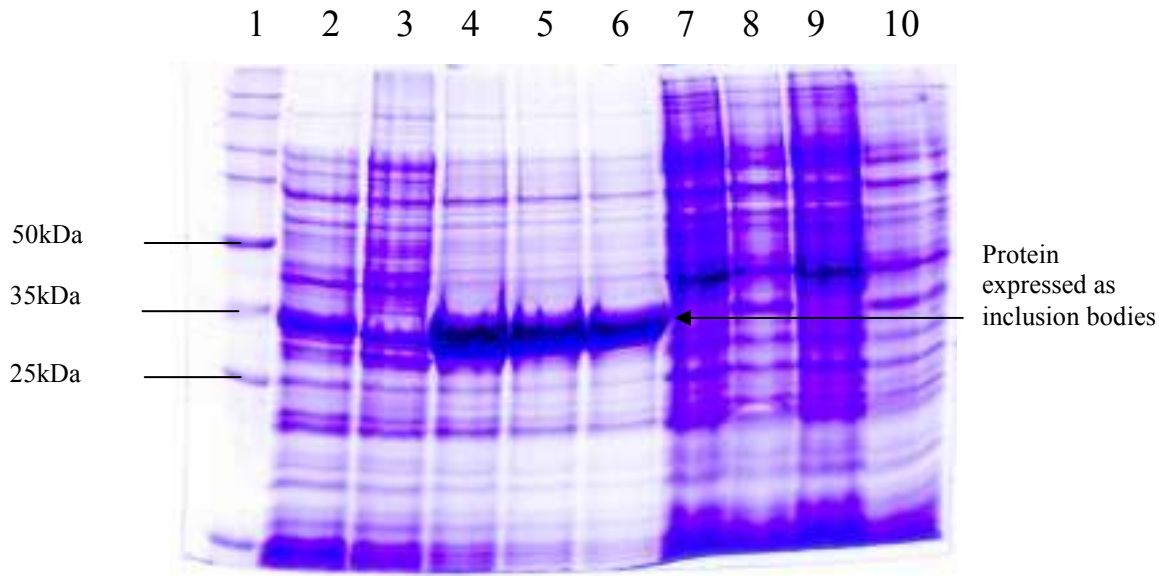


Fig 3.12 SDS-PAGE analysis illustrating formation of OtcX3 as inclusion bodies in in *E. coli* Tuner::DE3

Lane 1: 10µl Promega Broad Range marker

Lane 2: 15µl sample 2hr expression of pET33X3 construct in Tuner™::DE3

Lane 3: 15µl sample induced pET33b(+) vector after 2hrs

Lanes 4-6: 15µl samples of insoluble fractions of cell extract after lysis by French press of 2.5hr expression at 1, 0.5 and 0.25mM IPTG induction concentrations, respectively

Lanes 7 and 9: 30µl samples soluble fractions of expression at 1mM and 0.5mM IPTG induction concentrations, respectively

Lanes 8 and 10: as lanes 7/9, but 15µl samples

3.3 Attempted purification of OtcX3

OtcX3 was over-expressed almost entirely as inclusion bodies. Despite this, attempts were made to purify recombinant protein from the soluble fraction. A calibrated small-scale disposable Novagen column containing Ni^{2+}-charged His•bind resin (**Section 2.4.5**) was used to test a 50 ml lysate of *E. coli.* Results were sufficiently encouraging to upscale to 1L using *E. coli* Tuner::DE3 over-expressing OtcX3. Fractions that displayed UV absorbance at 280nm during elution were collected and analysed by SDS-PAGE.

Recombinant OtcX3 was eluted over six 2ml fractions (**Fig 3.13**) but fractions 25-27 were significantly contaminated with other proteins. Fractions 25-30 were concentrated by centrifugation at 3,000g, 4^{o}C using a Pall® micro-concentrator with a 10kDa filter to reduce the volume to 200µl. However, no protein was observed upon analysis by SDS-PAGE. It was assumed the protein had been degraded by a protease or was unstable in solution.

In an attempt to pool the eluted OtcX3 observed in **Fig 3.13** in a more efficient manner, the experiment was subsequently repeated by introducing empirical step gradients to the imidazole elution stage. This was done with a view to eliminating the competing proteins earlier, thus leaving the recombinant OtcX3 on the column. Remaining OtcX3 could then be eluted in a in a smaller volume and therefore negate the need for a further concentration step. Cellular matter built up visibly in the His•bind column.

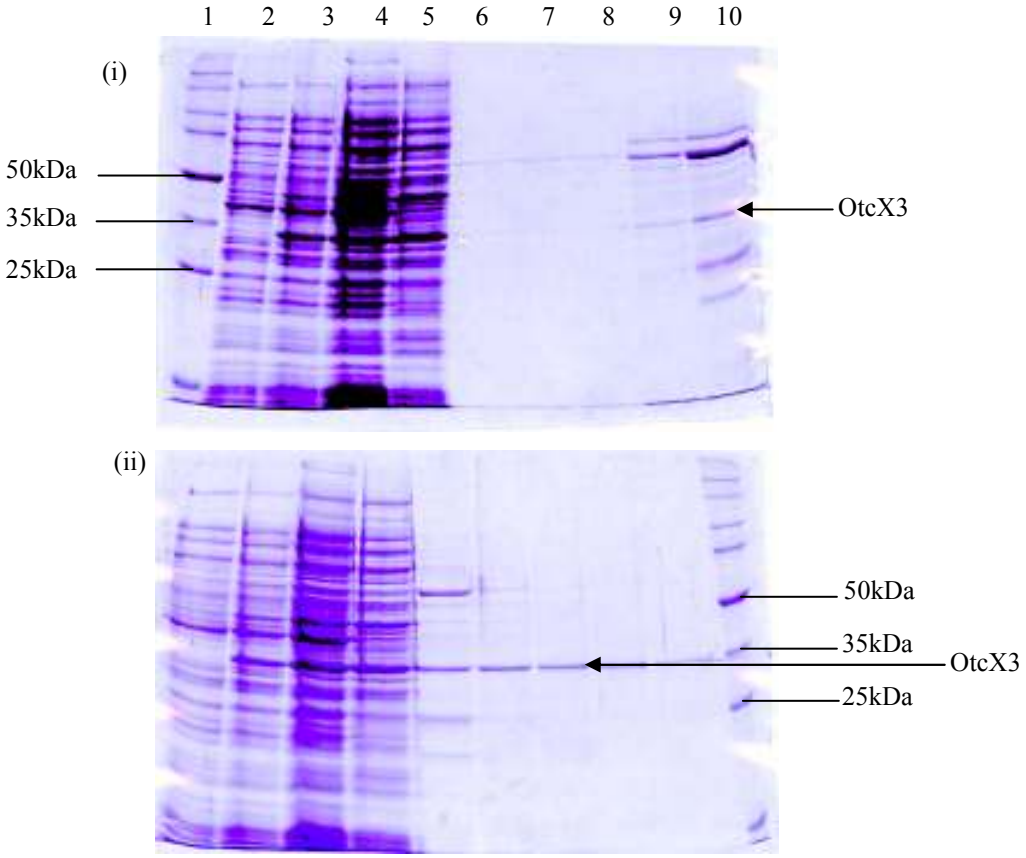


Fig 3.13 His•tag purification of OtcX3 using FPLC

Gel (i) Lane 1: 5µl Promega broad range marker

Lane 2: 15µl sample 2hr IPTG-induced negative control expression (pET33b(+)/no insert) in *E. coli* HMS174::DE3

Lane 3: as lane 2 but from 2hr expression of construct pET33X3

Lane 4: 15µl sample of soluble fraction of cell extract

Lane 5: 15µl sample of flow through

Lanes 6-10: 30µl samples of elution fractions 21-25 containing target protein

Gel (ii) Lane 1: 15µl sample 2hr IPTG-induced negative control expression (pET33b(+)/no insert) in *E. coli* HMS174::DE3

Lane 2: as lane 1 but with 2hr expression of construct pET33X3

Lane 3: 15µl sample of soluble fraction of cell extract

Lane 4: 15µl sample of flow through

Lanes 5-9: 30µl samples of elution fractions 26-30 containing target protein

Lane 10: 5µl Promega broad range marker

3.4 Solubilisation and refolding of OtcX3 from inclusion bodies

The *E. coli* Tuner™::DE3 strain was used as it gave the highest level of expression. Solubilisation of inclusion bodies by denaturants and/or detergents and subsequent refolding is a very empirical process. The formation of inclusion bodies from over-expression of heterologous genes in *E. coli* has been well documented (for reviews see Rudolph and Lilie, 1996; Clark, 1998; Misawa and Kumagai, 1999; Tsumoto *et al*., 2003). Denaturants such as 8M urea and 6M guanidine.HCl have been commonly used to solubilise inclusion bodies while the strong detergent Sodium Dodecyl Sulphate (SDS) and the weaker N-lauroylsarcosine (SLS) have been used less frequently.

The refolding process involves slow dialysis of the denaturing reagent out of solution and successful refolding is critically dependent on factors such as ionic strength, pH and conditions to favour formation of disulphide bonds. Partially-folded aggregates often form, which can drastically reduce the final yield of folded protein (Fink, 1998). Additives that encourage authentic bond formation such as L-cysteine/cystine and L-methionine, or amino acids that carry the sulphydryl (-SH) functional group combined with an oxidant e.g. oxidised glutathione, have been successful (Tsumoto *et al*., 1998). L-arginine can suppress aggregation of proteins out of solution although the reason has remained unclear (Arakawa *et al*., 2007).

Upon their isolation from a lysed pellet of *E. coli*, the sole benefit of refolding inclusion bodies is that they are usually relatively pure. Consequently, this facilitates any post-refolding purification procedures such as gel filtration or ion-exchange chromatography. Solubilisation and refolding of partially-purified inclusion bodies was initially attempted using 8M urea and subsequently 6M guanidine.HCl, as described in **Section 2.4.5**. However, the protein aggregated out of solution upon removal of the denaturant during dialysis when samples were analysed by SDS-PAGE.

Therefore, solubilisation of inclusion bodies was attempted using the weak detergent, N-lauroyl sarcosine (SLS), used for the successful refolding of an acetyl-transferase of animal fatty acid synthase (Rangan and Smith, 1996). SLS has a far weaker ionic strength than SDS. Instead of rapid solubilisation by 8M urea and 6M guanidine.HCl, the inclusion bodies are solubilised slowly in the presence of buffer containing 1-2% SLS (**Section 2.4.6)**. After solubilisation, the dialysis of the reagent out of solution creates a less extreme chemical change, thus encouraging the formation of correct folding patterns, which reduces the level of protein aggregation.

The progressive solubilisation of the protein from the inclusion bodies was monitored by measuring UV absorbance at 330nm over 90 minutes (**Fig 3.14)**. Absorption decreased steadily over the first 60 minutes before readings levelled off, indicating that the inclusion bodies had gone into solution. Insoluble material was subsequently removed by centrifugation of the sample at 3,000g for 10 min at 4^{o}C. The clarified supernatant was removed and transferred to pre-prepared dialysis tubing. SLS was then dialysed out of solution over 48 hours (**Section 2.4.7)**. Dialysed samples were concentrated to ~ 1ml using Pall® 10K microconcentrators and analysed by SDS-PAGE.

Fig 3.15 illustrated the difference in concentration of OtcX3 produced from *E. coli* Tuner™::DE3 compared to HMS174::DE3 being harvested after over-expressing OtcX3 for 3 hours. Analysis also indicated that the protein was still present in solution after 2-3 weeks. The solubilised protein was substantially pure, with much lower concentrations of other solubilised proteins present.

Size-exclusion chromatography (Sephadex G-200 beads) was used to separate and purify dialysed samples of OtcX3 from the remaining contaminating proteins (**Section 2.4.8**). Samples from dialysis were concentrated to ~1ml using a 10kDa micro-concentrator as described previously, before separation of the protein mixture by size exclusion at a flow rate of 0.07 ml.min^{-1}. A calibration kit (Sigma) was used to construct a logarithmic calibration curve with blue dextran (2,000kDa) as the void volume marker for the column (V_o) (22.9ml) (**Section 2.4.8)**. The volumes at

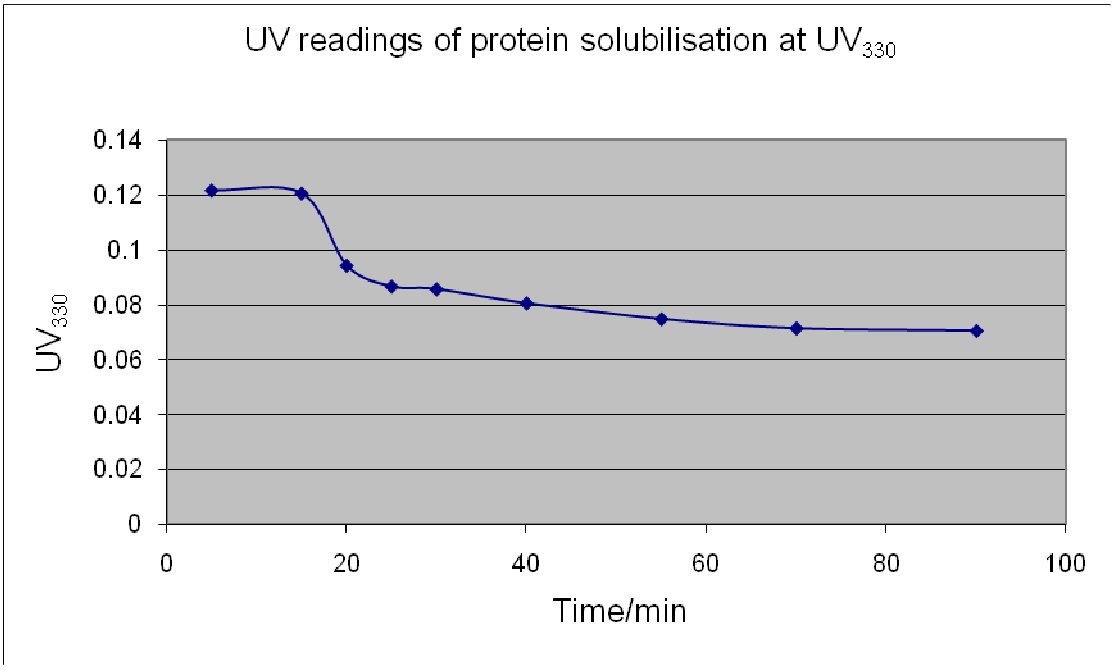


Fig 3.14 Graph illustrating UV absorption readings at 330nm during solubilisation of inclusion bodies isolated from *E. coli*.

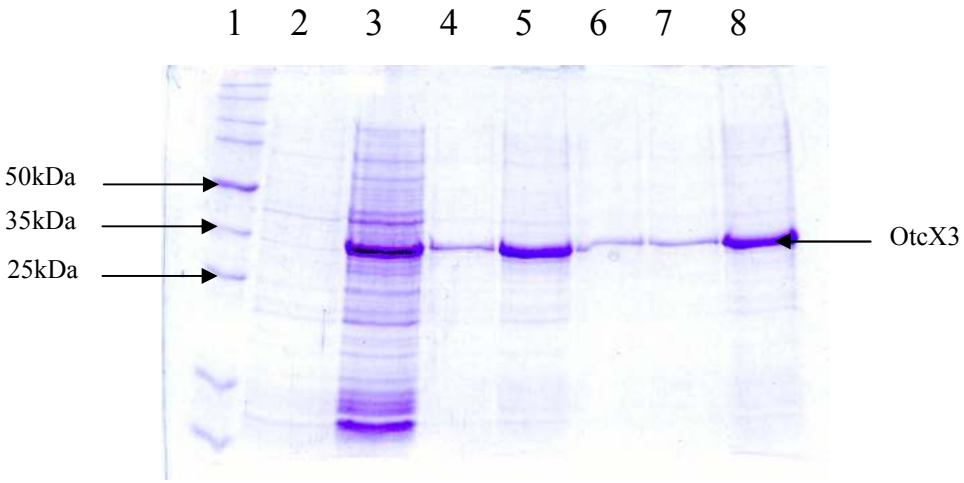


Fig 3.15 SDS-PAGE analysis illustrating samples taken from dialysed samples from expression in *E. coli* Tuner::DE3 and HMS 174::DE3

Lane 1: 5µl Promega Broad range marker

Lane 2: 15µl sample uninduced culture of Tuner/pET33X3

Lane 3: as lane 2 but induced culture after 2.5hrs

Lane 4: 30µl sample of solubilised inclusion bodies from HMS174::DE3

Lane 5: as lane 4 but for expression in Tuner::DE3

Lanes 6/7: as lane 4 but from 2 and 3 week old samples, respectively

Lane 8: as lane 5 but from 2 week old sample

which the calibrating proteins were eluted, V_e, were noted and V_e /V_o ratios plotted (**Fig 3.18; Table 3.1).**

A large fraction of the OtcX3 was eluted in the V_o and the remaining protein was eluted over fractions 28-34, peaking at 49.1ml. From **Fig 3.18,** this indicated the molecular weight of the protein was just over 30kDa, close to the hypothesized molecular weight of the protein of 35.7kDa. This result also indicated that the protein had a dimeric quaternary structure since each calibrating protein was a dimer, also. The fractions corresponding to the elution peak of the refolded, purified protein were concentrated using 10kDa microconcentrators and resuspended in storage buffer (**Section 2.4.5**). The UV spectra produced by the proteins used to calibrate the column were scanned and illustrated in **Fig 3.16**. **Fig 3.17** illustrated the UV spectrum from which refolded protein was eluted.

After storage at -20^{o}C for 48 hours, the sample of eluted protein could not be detected from analysis by SDS-PAGE. This indicated that the protein had been degraded by a protease or had aggregated out of solution. This was also observed in subsequent attempts to repeat the experimental procedures described above.

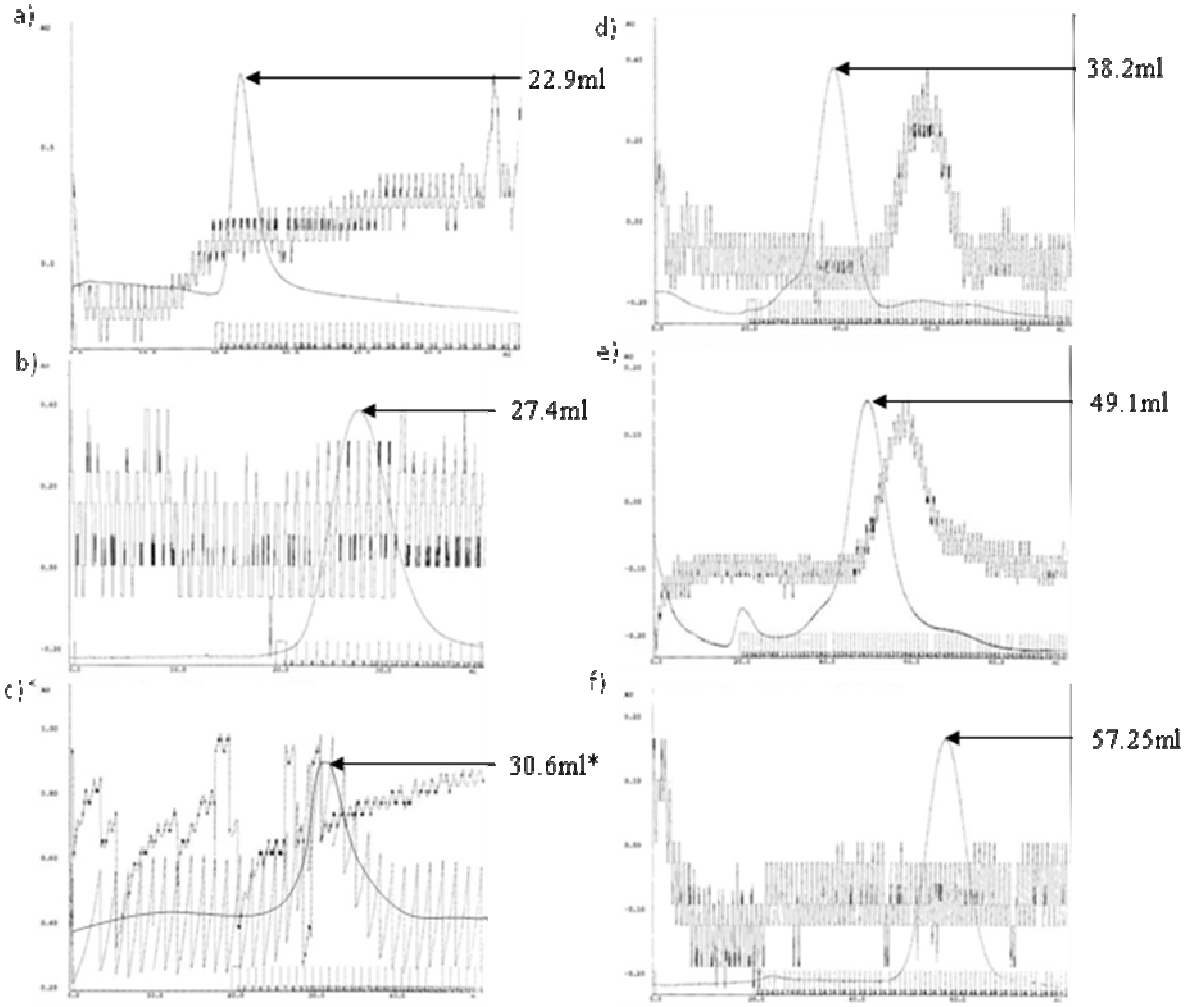


Fig 3.16 UV spectra at 280nm indicating elution volumes of calibration proteins

*Insoluble/unknown contaminant obstructing the UV sensor caused the absorption levels to jump in this sample, so the elution volume was highlighted by drawing a 'best fit' curve to indicate the UV absorption peak.

Protein	**Size (kDa)**	**V_e (ml)**	**V_e/V_o ratio**
Blue dextran (V_o)	2000	22.9	-
β amylase	200	27.4	1.2
alcohol dehydrogenase	150	30.6	1.33
BSA	66	38.2	1.67
carbonic anhydrase	29	49.1	2.14
cytochrome c	12.4	57.25	2.5

Table 3.1 Table summarising V_e/V_o ratios calculated from calibration of gel filtration column

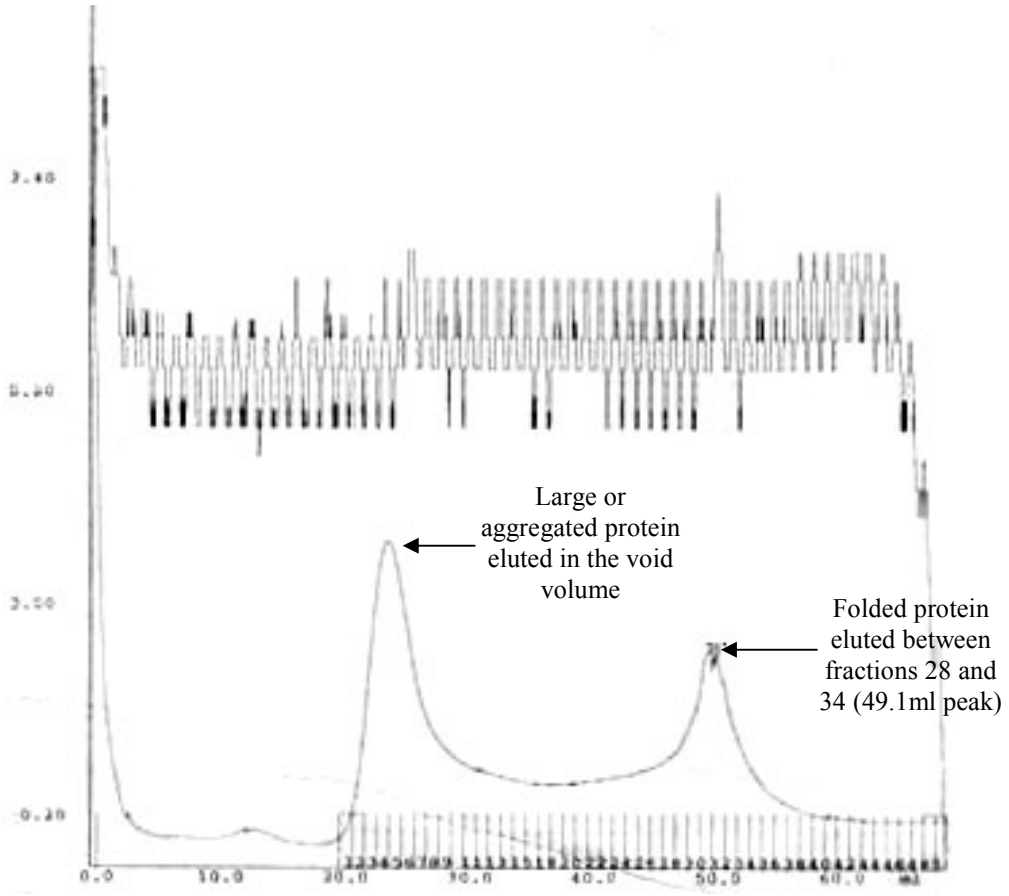


Fig 3.17 UV absorption spectrum at 280nm of dialysed samples containing refolded protein

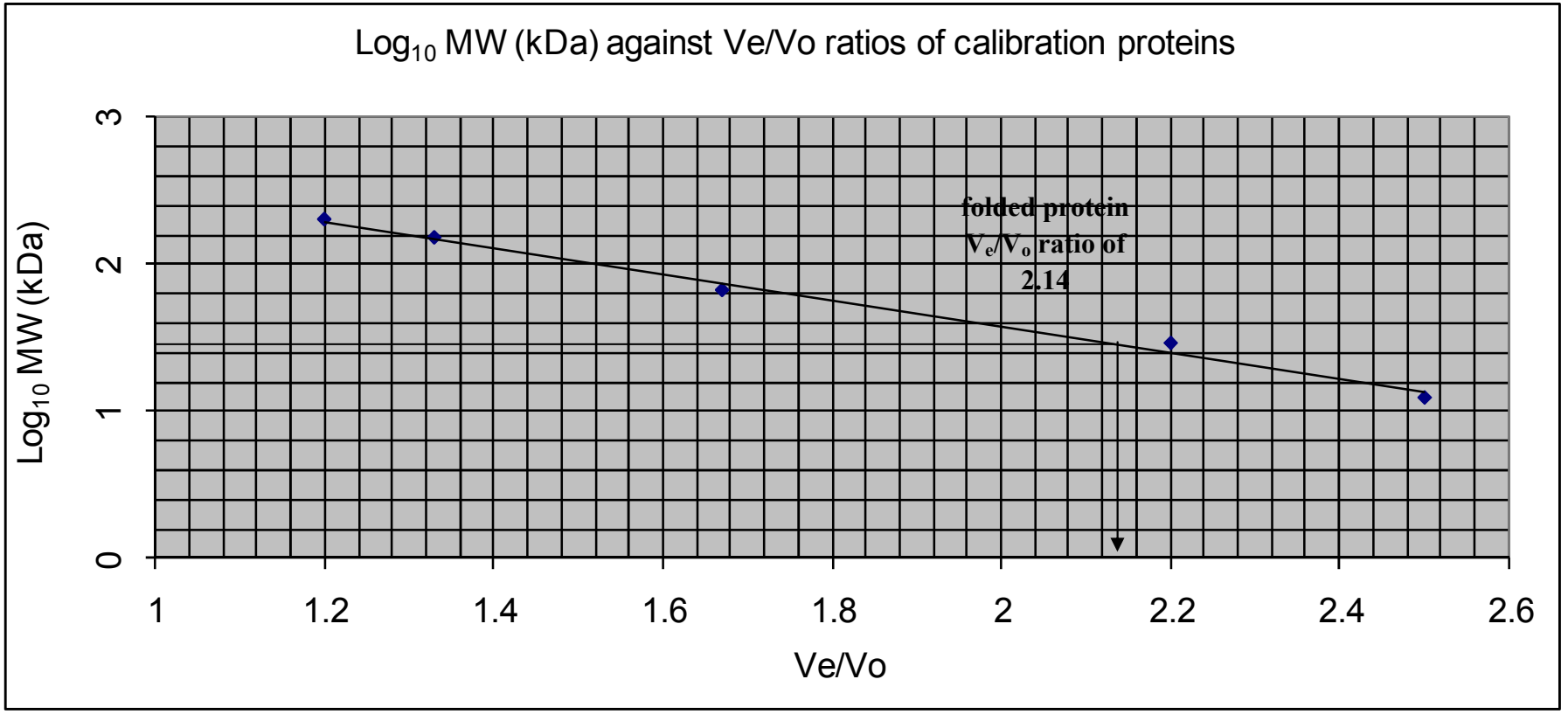


Fig 3.18 **Calibration curve from V_e/V_o ratios of standard proteins**

3.4 Conclusions

- Amplification and cloning of *otcX3* was successful. Over-expression of *otcX3* using IPTG induction of pET-driven system (Studier and Moffat, 1986) in *E. coli* DE3 lysogenic strains was also successful using the expression vector pET33b(+) but unsuccessful using pET15b. An explanation for this could not be established. Optimum conditions for over-expression were more dependent on the choice of strain than IPTG induction concentrations or growth temperature.
- Intracellular degradation of OtcX3 occurred after 3 hours in each strain tested indicating instability or proteolytic degradation of the recombinant protein. Analysis of cell lysates harvested after 3 hours indicated that recombinant OtcX3 over-expressed mainly as inclusion bodies. Efforts made to purify soluble recombinant OtcX3 showed initial promise (**Fig 3.13**) but degradation or aggregation of the protein out of solution occurred. Recurring technical problems with His•bind columns also hindered progress.
- Solubilisation and refolding of inclusion bodies using SLS as the denaturant followed by dialysis and size-exclusion chromatography indicated that OtcX3 existed as a dimer but degraded or aggregated out of solution soon after elution for reasons that were unclear but consistent with the rapid *in vivo* degradation observed.
- Biochemical analysis of OtcX3 could not be conducted using the strategies described.

Chapter 4:

PCR-Targeted Disruption of *otcX3*

4.1 The adaptation of PCR-targeted mutagenesis to *S. rimosus* M4018

The formation of recombinant OtcX3 as inclusion bodies in *E. coli* was described and discussed in **Chapter 3**. This meant that no soluble, active protein was available to conduct an assay. To explore further the function of *otcX3*, disruption of the chromosomal copy of *S. rimosus* M4018 was undertaken. This work was undertaken prior to the reports of Zhang *et al.,* 2006, 2006b, described below)

Heterologous co-expression of the *otc* minimal PKS genes in *S. coelicolor* CH999 (McDaniel *et al*., 1993), along with the amidotransferase, *otcY1-4*, produced a novel alkaloid with the carboxamido group incorporated (Zhang *et al*., 2006b) (**Fig 1.11**). When the same experiment was repeated with additional expression of *otcX3*, no enhanced incorporation of the carboxamido group was observed (Zhang *et al*., 2006). The disruption of *otcX3* described here is an adjunct to the information derived by Zhang's group.

The disruption of *otcX3* was attempted using an adaptation of a novel gene disruption protocol using targeted PCR products. The protocol, REdirect© Technology (Gust *et al*., 2003), designed for use with *S. coelicolor* A3(2) and initially implemented by Datsenko and Wanner (2000) for *E. coli*, was adapted for *S. rimosus* M4018.

Various methods of gene disruption have been available for research in *Streptomyces*, including the use of suicide vectors and *in vitro* transposition (Kieser *et al*., 2000). Datsenko and Wanner (2000) developed a new method of disrupting genes in *E. coli* using PCR products containing resistance markers, flanked by 39nt DNA sequences homologous to the gene sequence of interest. Gust *et al*., (2002; 2003) adapted this method for use in *S. coelicolor* A3(2) to form the REdirect© protocol by modifying these resistance cassettes to include the sequence of *oriT* (from vector RK2; Pansegrau *et al*., 1994). Cosmid DNA containing the *oriT* sequence could be transferred from *E. coli* to *Streptomyces* strains, by inter-species conjugation. Adaptation of this technique for *S. rimosus* was attractive, as a simple,

one-step protocol for creating mutagenized strains of *S. rimosus* M4018 to augment the genome sequence which was to be undertaken (Hunter, personal comm.).

To adapt REdirect© for *S. rimosus* M4018, vector DNA had to contain a contiguous sequence of DNA in which the gene of interest was located of sufficient lengths upstream and downstream to encourage homologous recombination, as frequency of recombination between two markers increases with distance (Sermonti and Carere, 1968). Two resistance markers were also required to positively select and subsequently screen and distinguish between a single and double crossover event. The sensitivity of the model organism, *S. coelicolor* A3(2), to a wide range of antibiotics was well documented (Hopwood *et al*., 1985); however, the same information was not available for *S. rimosus* M4018. The REdirect© cassettes initially available (JIC, Norwich, UK) conferred apramycin, streptomycin/spectinomycin and viomycin resistances, respectively (Gust *et al*., 2002). The sensitivity of *S. rimosus* M4018 to these and a variety of other antibiotics was tested on different media.

4.1.1 The sensitivity of *S. rimosus* M4018 to antibiotics on various media

Three types of media were tested: TSA, SFM and Emerson's (**Section 2.2.3**). Different media were used as the composition can have major effects on the efficacy of an antibiotic. Antibiotics used were: apramycin, streptomycin, spectinomycin, viomycin, gentamycin, kanamycin, erythromycin and hygromycin. 25ml plates were used as standard, containing a range of antibiotic concentrations. The plates were streaked with a loop (~10µl) of concentrated spore suspension of *S. rimosus* M4018 (**Section 2.2.5**) and incubated at 30^{o}C for several days. All antibiotics were prepared fresh and the growth on each plate was monitored daily.

Tables 4.1-4.3 summarised the sensitivity of *S. rimosus* M4018 to the antibiotics listed above when streaked on to SFM, Emerson's agar and TSA. Each (+) symbol represented an empirical level of growth observed on each plate after the noted time

of incubation at 30^{o}C. 1 point (+) represented weak growth (few colonies), while 4 points (++++) represented mature growth conferring resistance to the antibiotic.

For SFM (**Table 4.1**), the three immediately-available resistance cassettes (apramycin, viomycin and hygromycin) designed for REdirect©, would not be appropriate for *S. rimosus* M4018. There was only slight sensitivity to apramycin at a minimum concentration of 400μg.ml^{-1}. In the presence of viomycin and hygromycin, confluent growth was seen after 3 and 2 days growth, respectively. The latter was unexpected, as most *Streptomyces* are sensitive to hygromycin. High levels of resistance to streptomycin and spectinomycin were also observed. Kanamycin was tested at concentrations from 50-300μg.ml^{-1} with sensitivity displayed from 300μg.ml^{-1}. *S. rimosus* was sensitive to gentamycin from 200μg.ml^{-1} and erythromycin A was also shown to be selective from 40μg.ml^{-1}.

When tested on Emerson's agar, *S. rimosus* M4018 was found to be slightly more sensitive to the same antibiotics, in general (**Table 4.2**). Resistance was again observed to hygromycin, viomycin, streptomycin and spectinomycin. Sensitivity to apramycin was observed from 200μg.ml^{-1} compared to 400μg.ml^{-1} on SFM. Gentamycin was also selective from 100μg.ml^{-1} as opposed to 250μg.ml^{-1} on SFM. Kanamycin and erythromycin A also showed complete selectivity at 400 and 20μg.ml^{-1}, respectively.

TSA (Oxoid) was the most effective medium for selection of *S. rimosus* M4018. Apramycin showed selectivity from 100μg.ml^{-1}, while kanamycin and gentamycin were selective from 200μg.ml^{-1}. The effect of inoculum concentration was investigated further by spreading 10μl of *S. rimosus* M4018 spore suspension over TSA plates containing a range of concentrations of apramycin. As a control, this screening was conducted with *S. rimosus* M4018 containing the plasmid pG2 (**Section 2.1.2, Appendix A. 11**), a derivative of the *Streptomyces* integration plasmid, pSET152 (Bierman *et al*., 1992) that conferred apramycin and thiostrepton resistance.

Antibiotic / concentration (μg.ml^{-1})	Growth after 1 day	Growth after 2 days	Growth after 3 days	Growth after 7 days
No antibiotic (control)	+	++++	++++	++++
Apramycin / 50	-	++++	++++	++++
" / 100	-	+++	++++	++++
" / 200	-	+	++	++++
" / 400	-	-	-	+*
Viomycin / 200	-	+++	++++	++++
" / 300	-	+++	++++	++++
Hygromycin / 200	-	++++	++++	++++
" / 400	-	++++	++++	++++
Streptomycin / 200	-	++++	++++	++++
" / 400	-	++++	++++	++++
Spectinomycin / 200	-	++++	++++	++++
" / 400	-	++++	++++	++++
Gentamycin / 100	-	+++	++++	++++
" / 150	-	++	+++	++++
" / 200	-	+	++	+++
" / 250	-	-	-	+*
" / 300	-	-	-	+*
Kanamycin / 100	-	+++	++++	++++
" / 150	-	++	+++	++++
" / 200	-	+	+++	++++
" / 250	-	+	++	++++
" / 300	-	-	+	+++
Erythromycin A / 5	-	+	+++	++++
" / 10	-	-	+	++++
" / 20	-	-	+	++++
" / 40	-	-	-	+*

Table 4.1 Summary of sensitivity of *S. rimosus* M4018 to a selection of antibiotics on SFM

* maximum of 1-3 colonies grown after 7 days suggesting false positive therefore, it was concluded that the strain was sensitive to the antibiotic used at the noted concentration.

Antibiotic/ concentration (μg.ml^{-1})	Growth after 1 day	Growth after 2 days	Growth after 3 days	Growth after 7 days
No antibiotic (control)	+	++++	++++	++++
Apramycin / 50	-	++	++	++
" / 100	-	+	+	+
" / 200	-	-	-	-
" / 400	-	-	-	-
Viomycin / 200	-	+++	++++	++++
" / 400	-	+++	++++	++++
Hygromycin / 200	+	++++	++++	++++
" / 400	+	++++	++++	++++
Streptomycin / 200	-	++++	++++	++++
" / 400	-	++++	++++	++++
Spectinomycin / 200	-	++++	++++	++++
" / 400	-	++++	++++	++++
Gentamycin / 50	-	-	+	+
" / 100	-	-	-	-
" / 200	-	-	-	-
" / 400	-	-	-	-
Kanamycin / 50	-	++	++	++
" / 100	-	++	++	++
" / 200	-	+	++	++
" / 400	-	-	-	-
Erythromycin A / 10	-	-	-	+
" / 20	-	-	-	-
" / 40	-	-	-	-

Table 4.2 Summary of sensitivity of *S. rimosus* M4018 to a selection of antibiotics on Emerson's media

Antibiotic / concentration (μg.ml^{-1})	Medium	Growth after 4 days	Growth after 5 days	Growth after 7 days
Apramycin / 100	Trypticase Oxoid	+ -	++ -	++ -
" / 200	Trypticase Oxoid	- -	- -	+* -
" / 400	Trypticase Oxoid	- -	- -	- -
Gentamycin / 200	Trypticase Oxoid	-	-	-
" / 400	Trypticase Oxoid	-	-	-
Kanamycin / 200	Trypticase Oxoid	-	-	-
" / 400	Trypticase Oxoid	-	-	-

Table 4.3 Summary of sensitivity of *S. rimosus* M4018 to a selection of antibiotics on Oxoid and Trypticase TSA

* maximum of 1-3 colonies grown after 7 days suggesting false positive therefore, it was concluded that the strain was sensitive to the antibiotic used at the noted concentration

Fig 4.1 illustrated the difference that media composition can have on the resistance of *S. rimosus* M4018 to antibiotics. There was higher resistance to apramycin when streaked on SFM compared to TSA (**Fig 4.1a**). Further examples were shown (**Fig 4.1b)** with optimum selective concentrations for streaking using apramycin, gentamycin and kanamycin. The sensitivity of *S. rimosus* M4018 depended on inoculum concentration (**Fig 4.1c).** The presence of the *aac(3)IV* gene in pG2 led to uniform growth, regardless of the concentration of apramycin (**Fig 4.1d**). The sensitivity of *S. rimosus* M4018 to apramycin and kanamycin on TSA meant that the apramycin REdirect© cassette (*aac(3)IV-oriT,* Gust *et al*., 2003) could be used to disrupt *otcX3* and kanamycin could be used to distinguish between a single and double crossover event.

High levels of phosphate repress OTC production in *S. rimosus* (Behal *et al*., 1982; McDowall *et al*., 1999), as indicated by the production/loss of an orange-brown pigment. TSA is rich in phosphates, so OTC production was not observed. A dramatically-reduced phenotypic level of sporulation was observed for mature colonies. By comparing pigment production on SFM and Emerson's to TSA, any phenotypic effects related to OTC biosynthesis caused by disruption of *otcX3* could be observed.

A cosmid library of *S. rimosus* M4018 was constructed (Almuteirie, personal comm.) using the cosmid Supercos1 that encodes kanamycin resistance in *Streptomyces* (Stratagene, **Appendix A. 10**). This cosmid library was screened for cosmids containing the target gene, *otcX3* (**Section 4.2**) to comply with the methodology for the REdirect protocol.

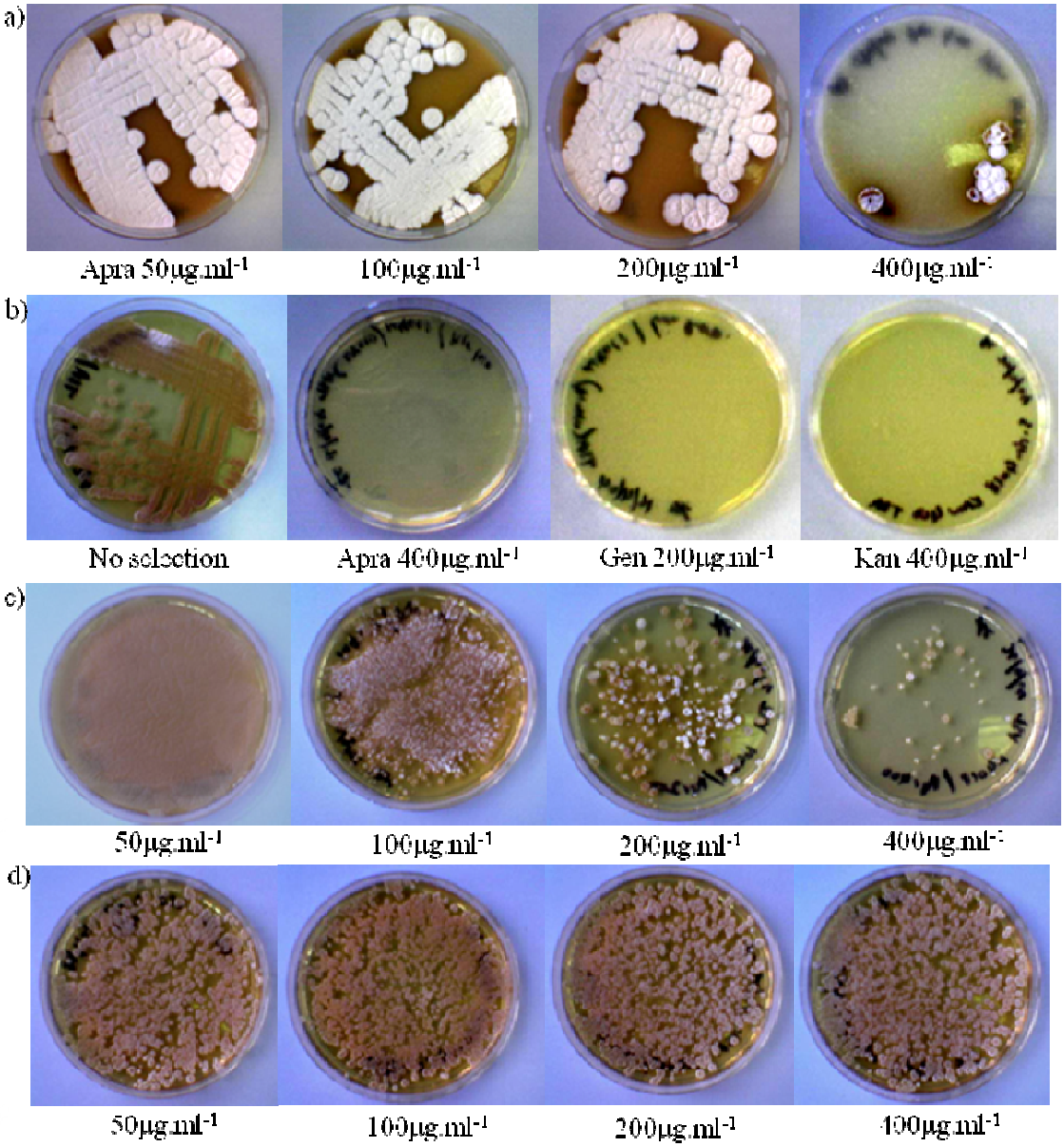


Fig 4.1 Sensitivity of *S. rimosus* M4018 to various antibiotics

a) streaked on SFM supplemented with apramycin (Apra), b) streaked on TSA supplemented with apramycin, gentamycin (Gen) and kanamycin (Kan), c) spread-plate inoculations on TSA supplemented with apramycin, d) as for c) except inoculated with *S. rimosus* M4018 containing plasmid, pG2

4.2 Hybridization of *otcX3* to a cosmid library of *S. rimosus* M4018

The REdirect© methodology required homologous flanking DNA upstream and downstream of *otcX3* but there was no construct available for this. A cosmid containing flanking contiguous sequences of homology was required.

A cosmid library of the *S. rimosus* M4018 genome had been constructed (Almuteirie, personal comm.) using Supercos1 (Stratagene). The first attempt at making this resulted in a lower number of clones than predicted. Around 750 colonies were obtained from transduction after the packaging of partially-digested fragments of *S. rimosus* chromosomal DNA inserts (30-40kb) into Supercos1 whereas 3,000 were deemed necessary for good statistical coverage. Nevertheless, these clones were used in eight 96-well plates to search for a clone containing *otcX,* using a DIG-labelled *otcX3* probe (**Section 2.3.12**).

A stock of *E. coli* DS941 (**Section 2.1.1**) containing pET33X3 was used as a positive control. *E. coli* containing Supercos1 only and another cosmid clone of *S. rimosus* M4018 known to lack *otcX3* (Almuteirie, personal comm.) were used as negative controls. Overnight hybridization at 42^{o}C to the DIG-labelled *otcX3* probe revealed two positive purple-blue signals upon development with NBT/BCIP dye detection (**Section 2.3.12**). The clones originated from plate A, position H6 (AH6, **Fig 4.2a**) and the other from plate E, position H1 (EH1, **Fig 4.2b**). The pET33X3 positive control and the negative control validated the filter.

To confirm the presence of *otcX3* within the identified cosmid clones, AH6 and EH1, cosmid DNA was isolated from overnight cultures and analysed by PCR (**Section 2.3.8**) and restriction digestion using the plasmids pET33X3 and pSRG3 (Garven, 1995) as positive controls. Supercos1 and a cosmid clone that did not hybridize to *otcX3* were used as negative controls. *OtcX3* was amplified from AH6 and EH1 (**Fig 4.3)**. The section of genomic DNA containing the *otcX* locus and *otcC* had been

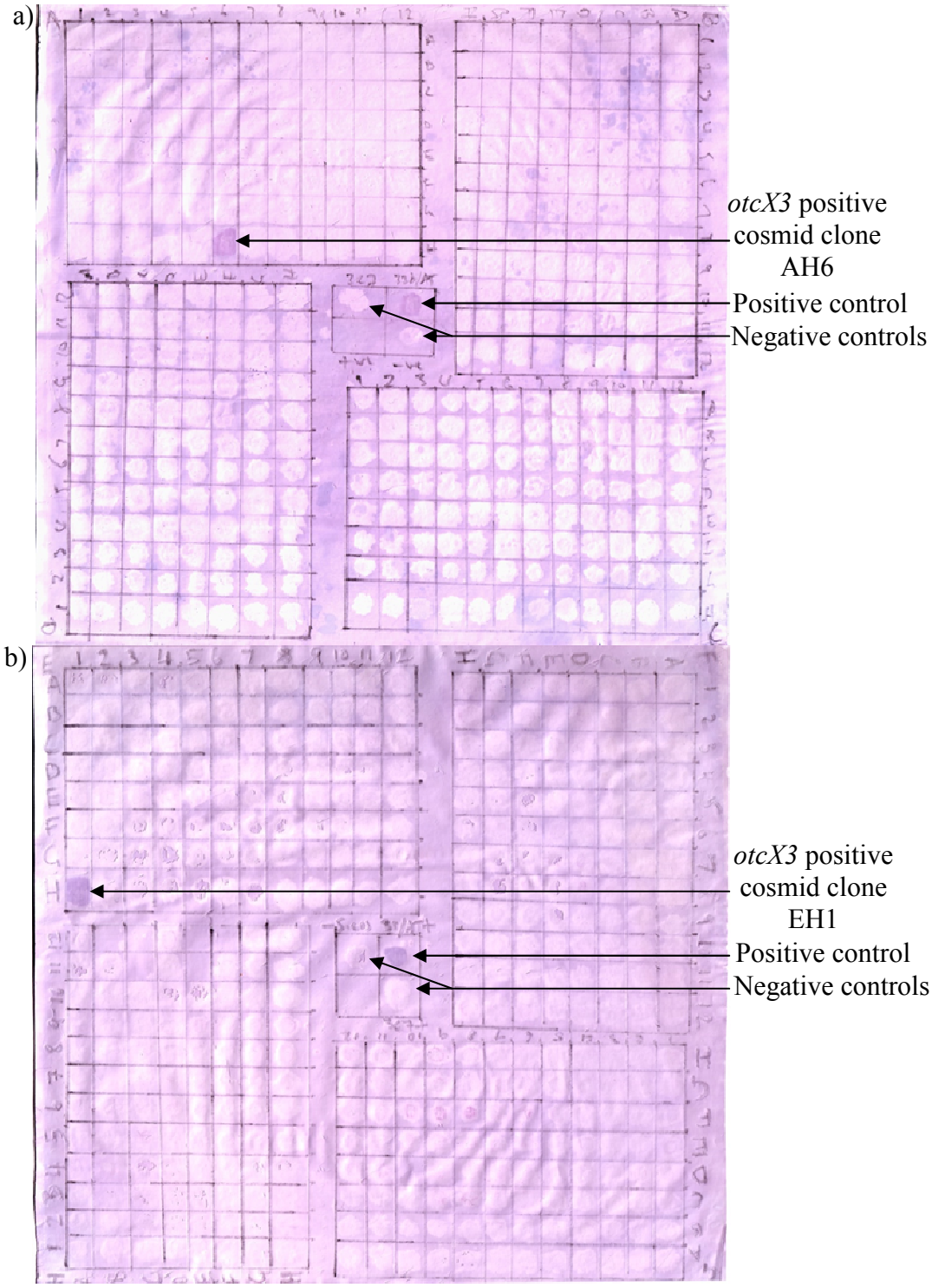


Fig 4.2 Colony lift hybridization of *otcX3* probe to cosmid library of *S. rimosus* M4018

a) Plates A-D (AH6 highlighted); b) Plates E-H (EH1 highlighted)

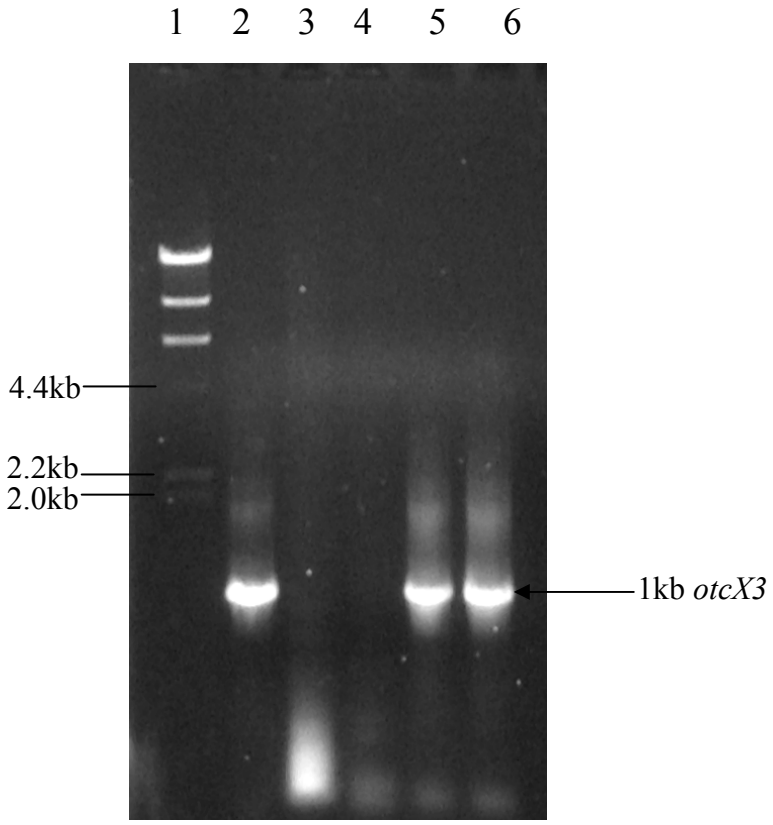


Fig 4.3 Amplification of *otcX3* from cosmids AH6 and EH1

Lane 1: 250ng λ *Hin*dIII marker (Promega)

Lane 2: positive control amplification of *otcX3* from pET33X3

Lane 3: negative amplification of *otcX3* from Supercos1

Lane 4: negative amplification of *otcX3* from cosmid 3C7

Lane 5: amplification of *otcX3* from cosmid AH6

Lane 6: amplification of *otcX3* from cosmid EH1

(all lanes 5µl of PCR product unless stated)

sequenced previously (Butler *et al*., 1989). This sequence contained a 5.2kb *Pst*I fragment, which was cloned to form the plasmid pSRG3 (Garven, 1995). Digestion of the cosmids with *Pst*I released this 5.2kb band. The 1.9kb *Sac*I fragment containing *otcX3* was also identified in the cosmids (**Fig 4.4**).

To assess the amount of DNA flanking *otcX3*, a 12kb *Sac*I fragment digested from EH1 corresponded to a known 12kb *Sac*I fragment in the *otc* cluster downstream from *otcX3* from *otcY2-1* to *otrR*, near the *otrB* gene (**Fig 1.9**) at the end of the cluster. This indicated that around 20kb of wild-type flanking DNA was located downstream of *otcX3* on EH1 and therefore, approximately 10-15kb upstream. The same 12kb *Sac*I fragment was not seen upon digestion of AH6. *Sac*I digestion revealed a larger band approximately 16-18kb in size. This indicated that approximately 9-11kb of the 12kb *Sac*I fragment was present downstream of *otcX3* on AH6, which, when combined with the 7kb of Supercos1, would produce a fragment of the size seen in the *Sac*I digestion of AH6 (**Fig 4.4**). This meant that sufficient flanking wild-type DNA existed upstream of *otcX3* on AH6 to encompass the *otrA* gene, which was subsequently confirmed on both AH6 and EH1, while hybridization to *otrB* was confirmed in EH1, but not in AH6 (Almuteirie, personal comm.).

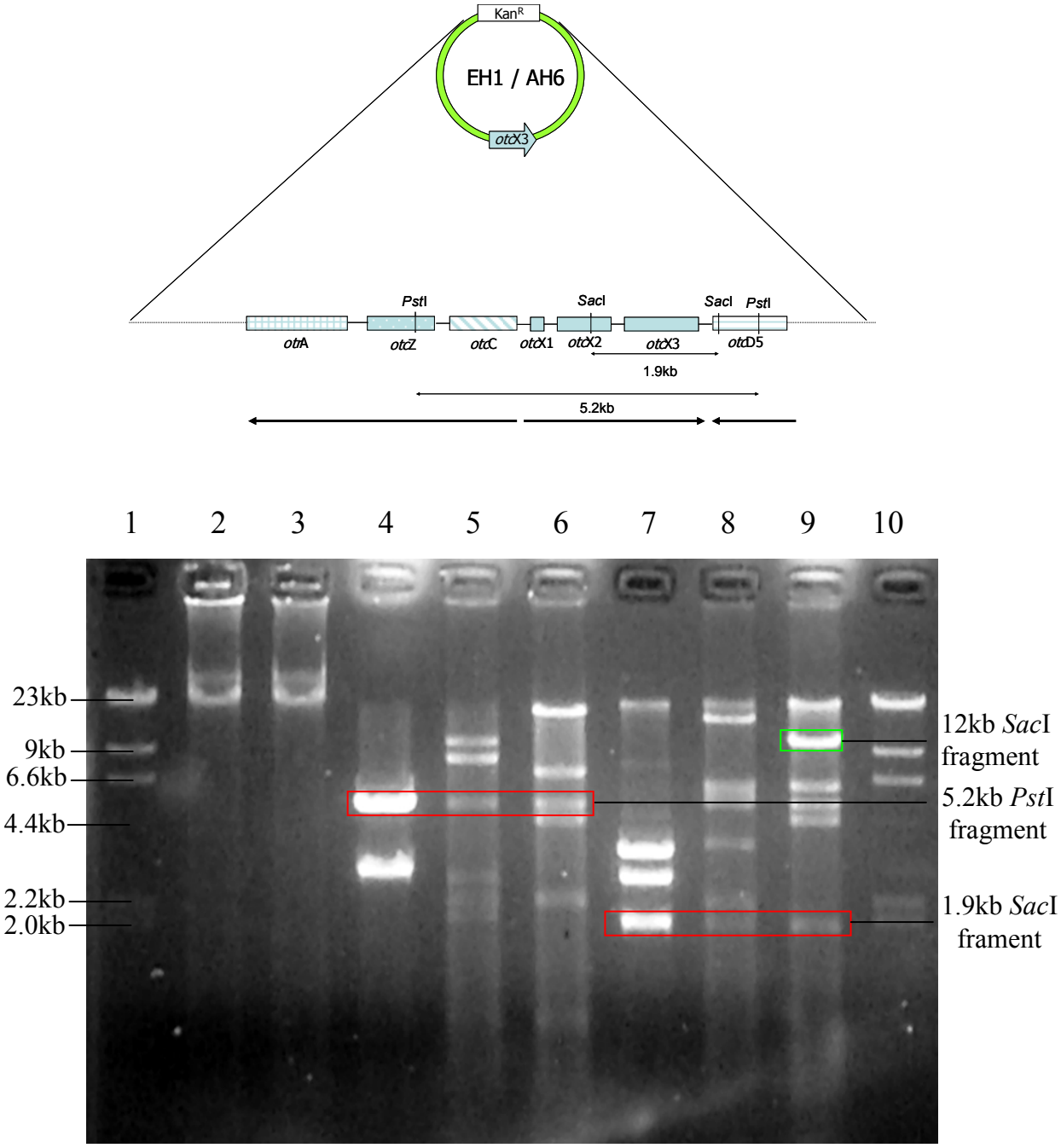


Fig 4.4 Restriction digestion of AH6 and EH1 with *Pst*I and *Sac*I

Lanes 1 and 10: 125ng λ *Hin*dIII DNA marker; Lane 2: 50ng uncut cosmid AH6; Lane 3: 50ng uncut cosmid EH1; Lane 4: 150ng pSRG3 digested with *Pst*I; Lane 5: 100ng AH6 digested with *Pst*I; Lane 6: 100ng EH1 digested with *Pst*I; Lane 7: 150ng pSRG3 digested with *Sac*I; Lane 8: 100ng AH6 digested with *Sac*I; Lane 9: 100ng EH1 digested with *Sac*I.

4.3 REdirect© PCR-targeted disruption of *otcX3*

4.3.1 Primer design and optimisation of PCR

Following the results discussed above, the *aac(3)IV-oriT* cassette from the plasmid pIJ773 (Gust *et al*., 2002, **Appendix A. 9**) encoding apramycin resistance was chosen for the PCR-targeted mutagenesis of *otcX3* (**Fig 4.8**). Primers were therefore designed to synthesise a cassette that could replace *otcX3* on the cosmids, AH6 and EH1 (**Section 4.2)** based on the known sequence of the *otc* cluster. The PCR reaction was thus optimised and used to synthesise the 'disruption cassette' (**Section 2.3.9)**.

Difficulties were encountered while attempting to amplify the *aac(3)IV-oriT* cassette using the purified cassette as a template. After amplification of the *aac(3)IV-oriT* cassette using pIJ773 as the template, *Dpn*I was added to the PCR product to digest and remove methylated pIJ773 DNA to minimise the number of false positives after electroporation. The amplified disruption cassette was then purified from an agarose gel (**Section 2.3.6**) and suspended in a minimum volume of dH_2O.

4.3.2 Disruption of *otcX3* in *E. coli* BW25113

E. coli BW25113 containing the λ Red plasmid, pIJ790, was transformed with AH6 and EH1 selecting with ampicillin, kanamycin and chloramphenicol at 30^{o}C (to retain the temperature-sensitive pIJ790). Transformants were then used to prepare electrocompetent cells from overnight cultures (**Section 2.2.10**). Sterile L-arabinose was added to the growing culture at a final concentration of 10mM to induce expression of the λ Red genes. 1μl (~100ng) purified disruption cassette was then used to transform the electrocompetent cells.

Cosmid DNA was isolated from ampicillin, kanamycin and apramycin resistant *E. coli* BW25113, growing cultures with apramycin to eliminate any wild-type copies of cosmid DNA. PCR and restriction analysis was then conducted to confirm the disruption of *otcX3* on the respective cosmids. **Fig 4.5** showed the amplification of

the *aac(3)IV-oriT* cassette on the disrupted cosmid DNA and its concurrent absence from the negative controls.

Fig 4.6 shows the restriction map of the region containing *otcX3* and the hypothesized changes that would occur in the map upon double crossover recombination between the disruption cassette and *otcX3*, which is located within a 1.884kb *Sac*I fragment. The REdirect© primers were designed to target and replace the complete 1.031kb *otcX3* sequence with the 1.384kb *aac(3)*IV-*oriT* cassette. If successful, the size of the mutagenized *Sac*I fragment would therefore increase by 361bp from the wild-type sequence to 2.245kb. The *aac(3)IV-oriT* cassette from pIJ773 (**Appendix A. 9**) had two internal *Sac*I sites that released a 751bp fragment within the *aac(3)IV* gene itself. Digestion with *Sac*I on the mutagenized cosmids would therefore split the enlarged 2.245kb fragment into 3 fragments; the 751bp fragment internal to *aac(3)IV-oriT*, a smaller fragment of 252bp downstream of the *aac(3)IV-oriT* cassette, and the remaining 1.242kb fragment upstream of *aac(3)IV* containing the *oriT* sequence and part of *otcX2*. Insertion of the *aac(3)IV-oriT* cassette would introduce a new *Pst*I site on the 5'-FRT sequence, which would increase the size of the wild-type ~5.2kb *Pst*I fragment by 361bp to a mutagenized size of ~5.56kb that would subsequently split into 3.45kb and 2.1kb fragments after digestion (**Fig 4.6**).

*Pst*I restriction analysis of the wild-type and mutagenized cosmids (**Fig 4.7)** shows that the original 5.2kb wild-type fragment was absent from the mutagenized cosmids. The 3.45kb and 2.1kb fragments were observed with digested mutagenized EH1, but the 2.1kb fragment was not distinguishable in mutagenized AH6. This was assumed to be caused by masking of that fragment by another *Pst*I fragment of similar size on the wild-type cosmid. Digested pSRG3 (Garven, 1995) was used as a control.

*Sac*I restriction analysis of the wild-type and mutagenized cosmids (**Fig 4.8)** shows that the 1.884kb *Sac*I fragment was cleaved in both wild-type cosmids just beneath the 2.0kb standard marker. Digestion of the mutagenized cosmids cleaved the hypothesized 1.242kb and 751bp fragments that did not exist in the wild-type

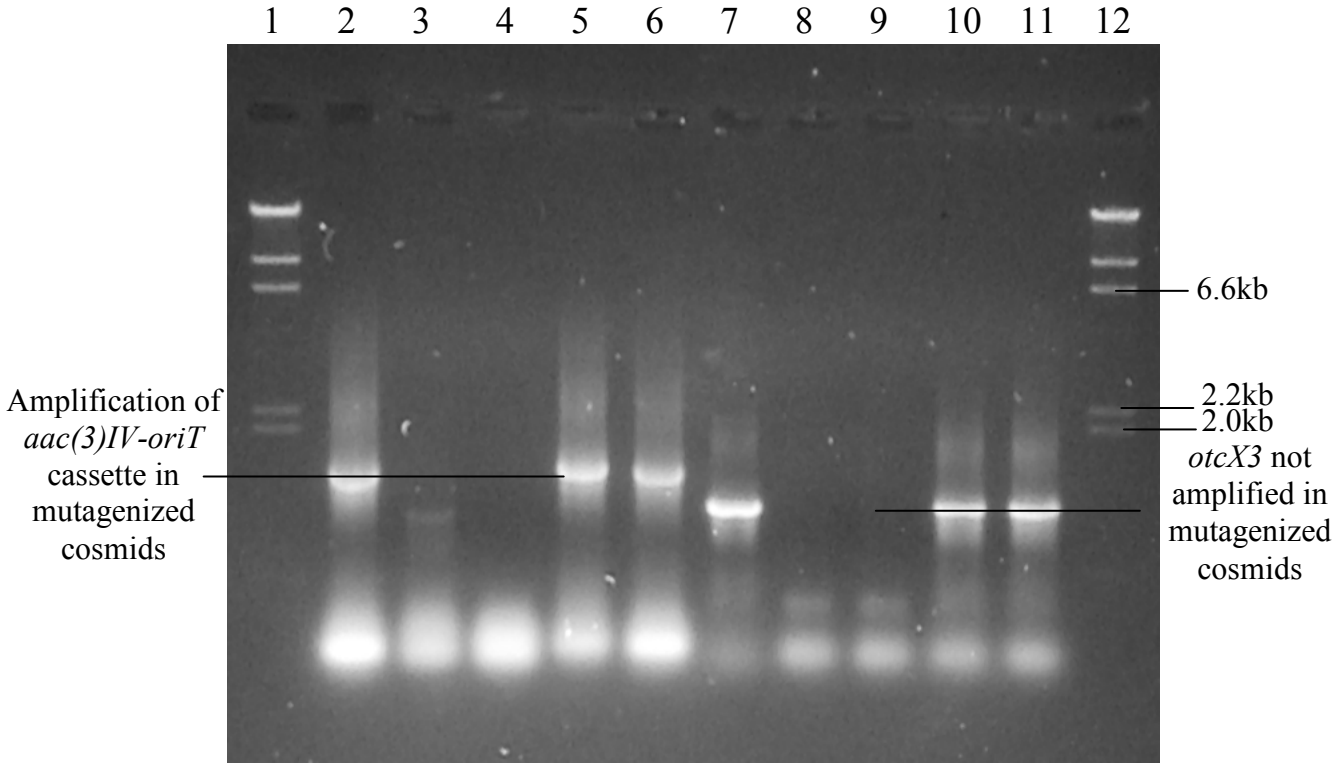


Fig 4.5 Amplification of *aac(3)IV-oriT* cassette in mutagenized cosmids

(All lanes 5µl, unless stated)

Lane 1 and 12: 125ng λ *Hin*dIII DNA marker

Lane 2: positive control amplification of *aac(3)IV-oriT* cassette from pIJ773

Lane 3: negative amplification of *aac(3)IV-oriT* cassette from cosmid, 3C7

Lane 4: negative amplification of *aac(3)IV-oriT* cassette from Supercos 1

Lane 5: amplification of *aac(3)IV-oriT* cassette from AH6Δ*otcX3*::*aac(3)IV-oriT*

Lane 6: amplification of *aac(3)IV-oriT* cassette from EH1Δ*otcX3*::*aac(3)IV-oriT*

Lane 7: positive control amplification of *otcX3* from pSRG3

Lane 8: negative amplification of *otcX3* from AH6Δ*otcX3*::*aac(3)IV-oriT*

Lane 9: negative amplification of *otcX3* from EH1Δ*otcX3*::*aac(3)IV-oriT*

Lane 10: positive control amplification of *otcX3* amplification from AH6

Lane 11: positive control amplification of *otcX3* amplification from EH1

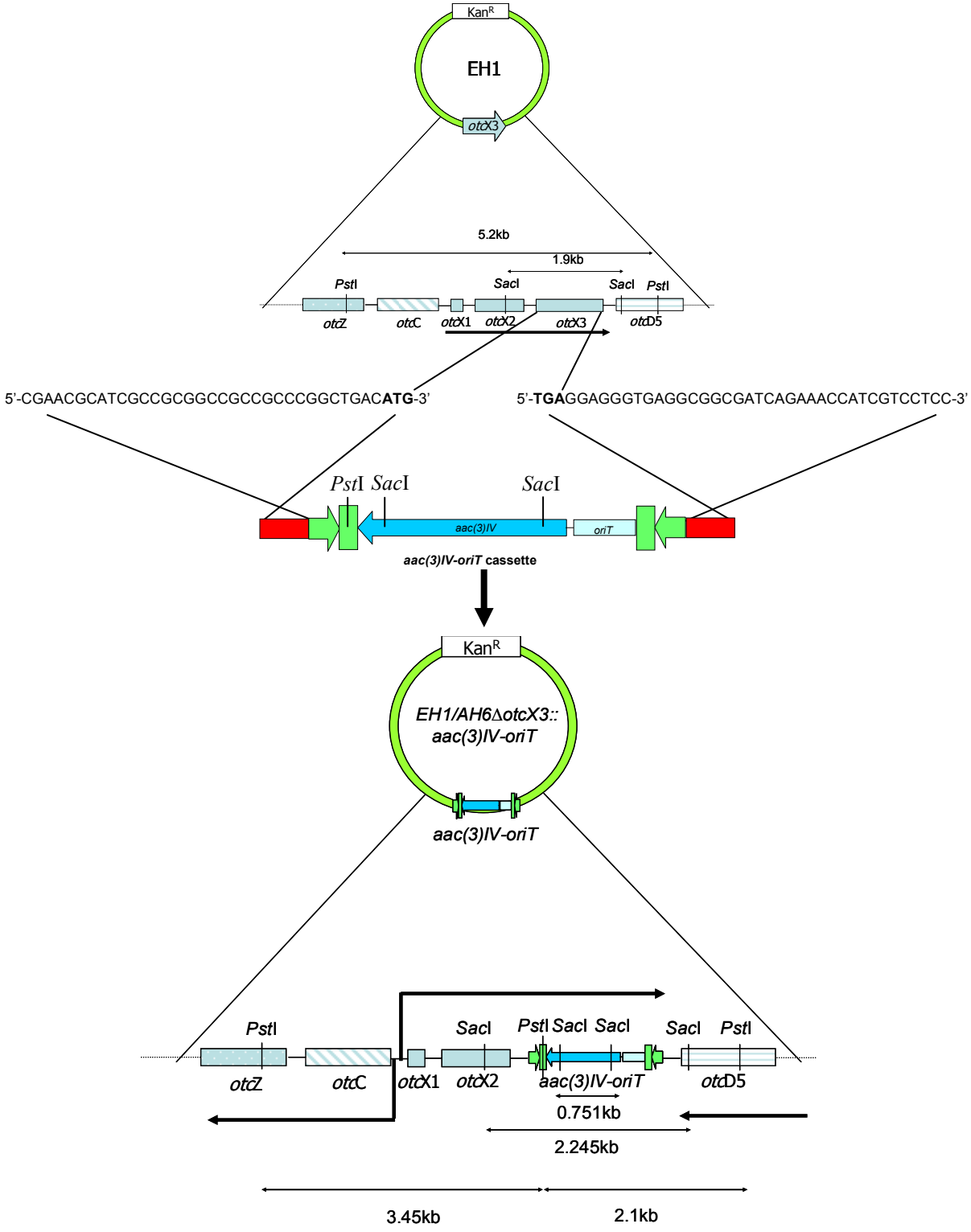


Fig 4.6 **Restriction map illustrating change inflicted by disruption of *otcX3* with *aac(3)IV-oriT* in *E. coli***
Thick arrows indicate promoters/direction of transcription

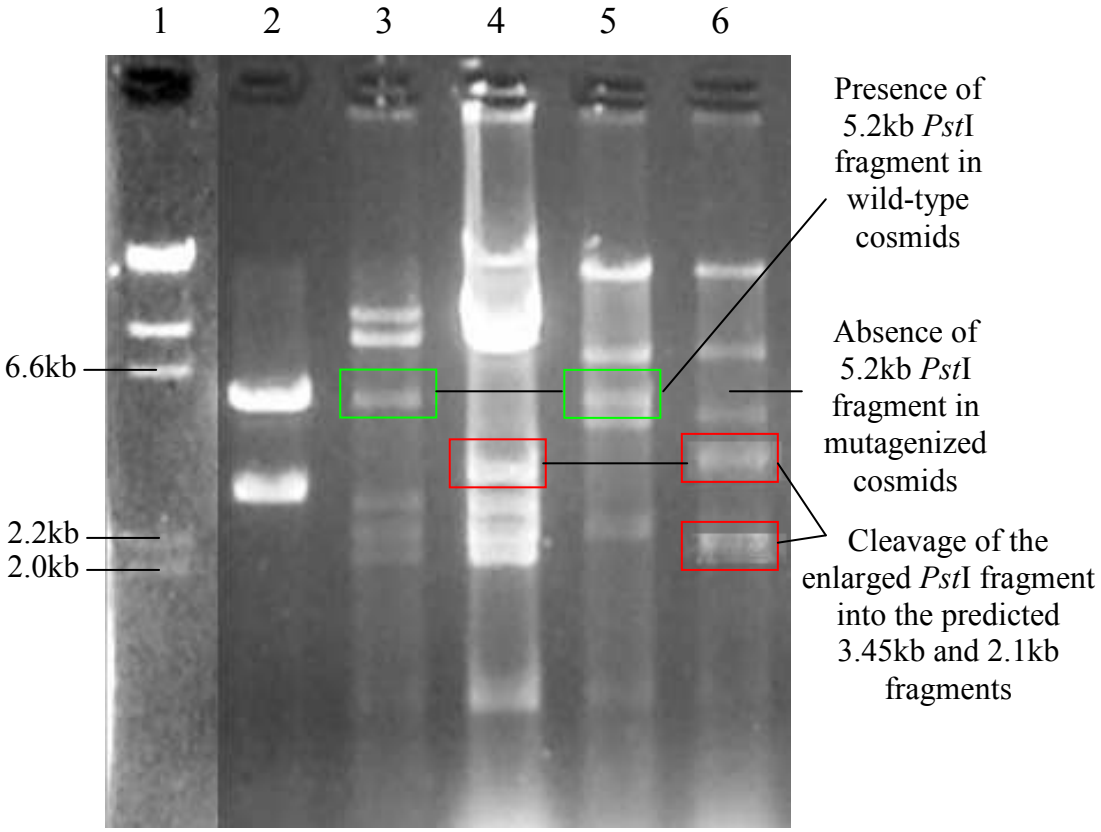


Fig 4.7 Restriction digestion of wild-type and disrupted cosmids using *Pst*I (altered areas enhanced using Adobe Photoshop to make bands of interest more visible)

Lane 1: 125ng λ *Hin*dIII DNA marker

Lane 2: 150ng pSRG3 positive control for 5.2kb wild-type fragment digested with *Pst*I

Lane 3: 75ng cosmid, AH6, digested with *Pst*I

Lane 4: 150ng AH6Δ*otcX3*::*aac(3)IV-oriT* digested with *Pst*I

Lane 5: 75ng cosmid, EH1, digested with *Pst*I

Lane 6: 50ng EH1Δ*otcX3*::*aac(3)IV-oriT* digested with *Pst*I

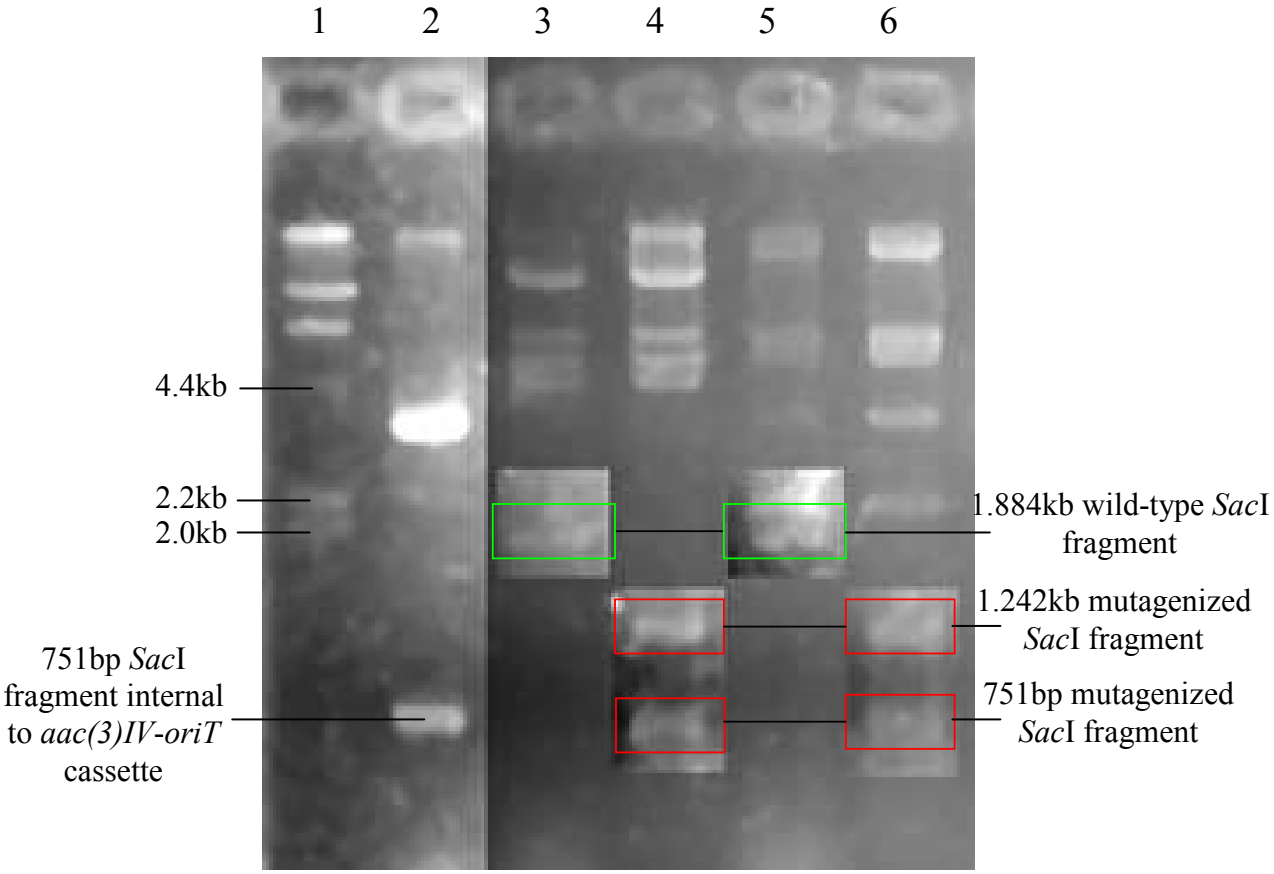


Fig 4.8 Restriction digestion of wild-type and mutagenized cosmids with *Sac*I (altered areas enhanced using Adobe Photoshop to make fragments of interest more visible)

Lane 1: 125ng λ *Hin*dIII DNA marker

Lane 2: 100ng pIJ773 positive control for 751bp internal *aac(3)IV-oriT* fragment digested with *Sac*I

Lane 3: 50ng cosmid EH1 digested with *Sac*I

Lane 4: 100ng EH1Δ*otcX3*::*aac(3)IV-oriT* digested with *Sac*I

Lane 5: 40ng cosmid AH6 digested with *Sac*I

Lane 6: 100ng AH6Δ*otcX3*::*aac(3)IV-oriT* digested with *Sac*I

sequence but the 252bp fragment was not visible on a 0.8% agarose gel. Digestion of pIJ773 was used as a positive control for identification of the internal 751bp *Sac*I fragment of the *aac(3)IV-oriT* cassette. In conclusion, PCR and restriction digestion analyses confirmed that the disruption of *otcX3* on the cosmids had been successful.

4.3.3 Electroporation of *S. rimosus* M4018 with mutagenized cosmids isolated from *dam⁻ dcm⁻ E. coli*

The *dam⁻ dcm⁻ E. coli* ET12567 strain (MacNeil *et al*., 1992) containing the non-transmissible plasmid pUZ8002 (Paget *et al*., 1999) was transformed with the mutagenized cosmids AH6Δ*otcX3*::*aac(3)IV-oriT* and EH1Δ*otcX3*::*aac(3)IV-oriT*. Transformants were selected with choramphenicol, kanamycin, ampicillin and apramycin and demethylated cosmid DNA was subsequently isolated from overnight cultures. Initially, conjugation was attempted, hence the presence of the driver plasmid, pUZ8002. Conjugation was unsuccessful however, thus electroporation of lysozyme-treated *S. rimosus* M4018 mycelium was undertaken.

1µl (~50ng.µl^{-1} in dH_2O) cosmid DNA was then used to transform lysozyme-treated *S. rimosus* M4018 mycelium (**Section 2.2.11** and **2.2.14**) (Pigac and Schrempf, 1995). Transformants were selected with apramycin at 200 and 400µg.ml^{-1}. The plasmid, pG2, encoding apramycin and thiostrepton resistance was used to transform *S. rimosus* M4018 as a control. Apramycin was used at different concentrations to investigate the difference in background growth that was likely to occur after the observations made from screening analysis (**Section 4.1)**, which illustrated that the concentration of inoculum was a critical factor that affected the sensitivity of *S. rimosus* M4018 to apramycin.

After 48hrs incubation at 30^{o}C, examination of plates showed background growth as very small colonies growing amongst larger transformants that conferred true apramycin resistance. Heavy background growth was observed on plates that were inoculated with higher concentrations of mycelium at 200µg.ml^{-1} apramycin, which confirmed that future transformations would be conducted with selection at a

minimum of 400μg.ml^{-1} apramycin. 104 putative recombinants (not including pG2 control) were identified; 69 were patched from transformation using AH6Δ*otcX3*::*aac(3)IV-oriT* and 35 from EH1Δ*otcX3*::*aac(3)IV-oriT*. These were patched onto three separate sets of TSA plates containing apramycin (400μg.ml^{-1}), kanamycin (250μg.ml^{-1}) and thiostrepton (10μg.ml^{-1}), respectively. Single crossovers were resistant to apramycin and kanamycin while double crossovers were resistant to apramycin and sensitive to kanamycin. Approximate transformation efficiencies for each electroporation were calculated; 2.56 x 10^5 cfu.μg^{-1} DNA for transformation with AH6Δ*otcX3*::*aac(3)IV-oriT*, 1.29 x 10^5 cfu.μg^{-1} for EH1Δ*otcX3*::*aac(3)*IV-*oriT* and 1.19 x 10^5 cfu.μg^{-1} for pG2. A diagram illustrating the possible outcomes from recombination between AH6/EH1Δ*otcX3*::*aac(3)IV-oriT* and the chromosome of *S. rimosus* M4018 was illustrated in **Fig 4.9**.

4.3.4 Phenotypic analysis of putative mutants

After 48hrs growth, phenotypic differences could be observed on the patched recombinants. In *S. coelicolor* A3(2), double:single crossover frequency ratios have been shown to be around 1:10 when introducing the mutagenized cosmid to the wild-type strain by conjugation (Herron, personal comm.). Of the 104 putative mutants isolated in this instance, all putative mutants displayed the phenotype of a double crossover event except for one. **Fig 4.10a-c** illustrated the phenotypes of the patched putative mutants on TSA, containing apramycin, kanamycin and thiostrepton as a negative control, respectively. Single colonies were then picked and streaked on SFM and Emerson plates selecting with and without apramycin to investigate whether any phenotypes were induced from expression of the *aac(3)IV* gene.

Most of the putative mutants displayed a slightly darker brown phenotype compared to the wild-type and growth on media containing apramycin darkened this colour and also showed evidence of reduced sporulation levels. It is likely that the reduced sporulation was caused by the extra stress of growth under antibiotic selection. Some of the putative mutants ($AH6_{200}$ 28, $AH6_{400}$ 28) produced an almost black phenotype

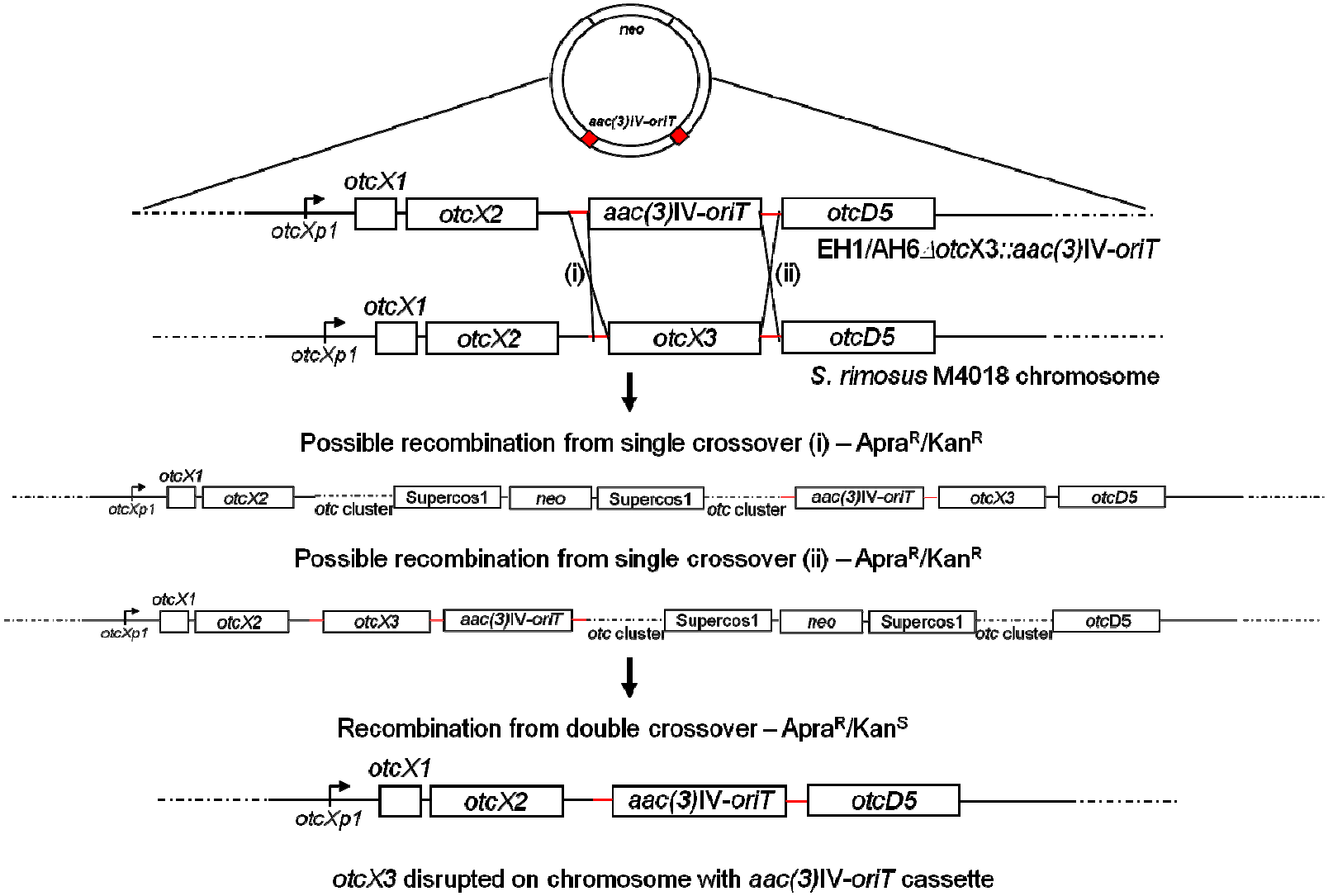


Fig 4.9 Possible recombination events between mutagenized cosmids and *S. rimosus* M4018 chromosome

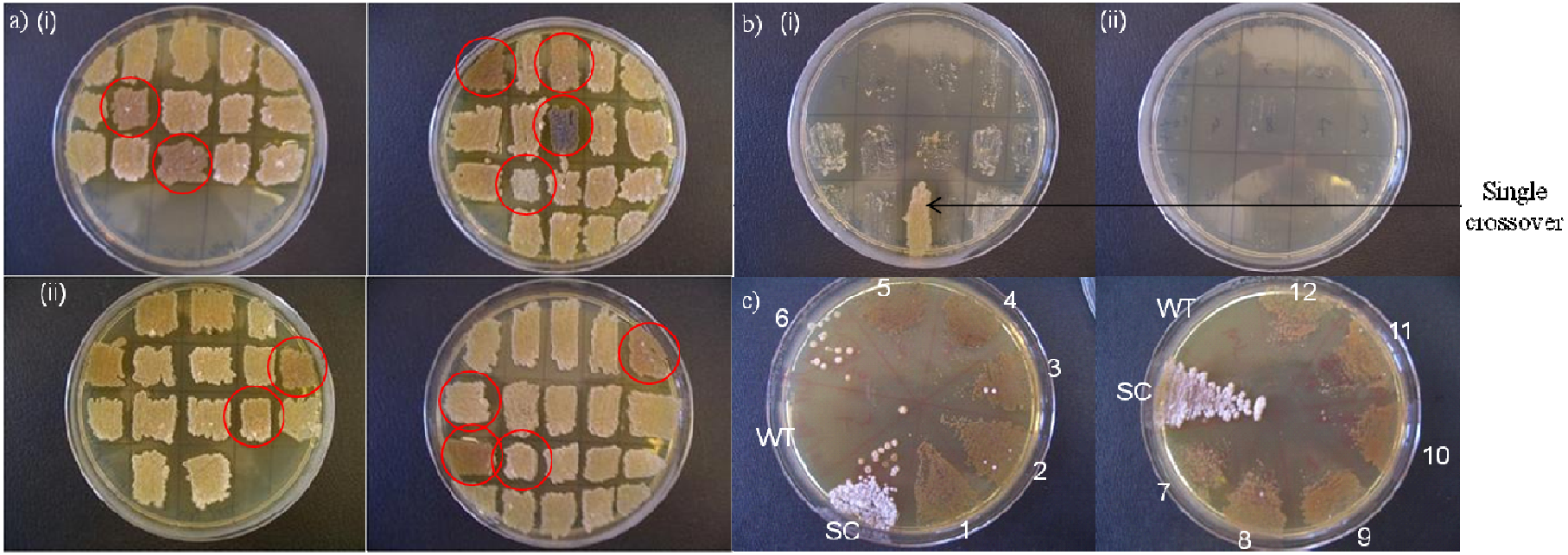


Fig 4.10 Phenotypic analysis of putative mutants

a) Selected mutants from transformation of *S. rimosus* M4018 with (i) AH6Δ*otcX3*::*aac(3)IV-oriT* and (ii) EH1Δ*otcX3*::*aac(3)IV-oriT*. Grown on TSA supplemented with aparamycin at 400µg.ml^{-1}. Putative mutants circled in red chosen for further analysis.

b) Putative mutants grown on TSA with (i) kanamycin at 250µg.ml^{-1} and (ii) selecting with thiostrepton at 10µg.ml^{-1} as a control. Single crossover highlighted.

c) Growth of selected putative mutants on TSA supplemented with apramycin and kanamycin at 400µg.ml^{-1} and 400µg.ml^{-1}, respectively. Key: WT – *S. rimosus* M4018 wild-type; SC – putative single crossover; 1-6 - putative mutants from transformation with AH6Δ*otcX3*::*aac(3)IV-oriT*; 7-12 – EH1Δ*otcX3*::*aac(3)IV-oriT*.

while the only recombinant not to show any pigment production was $AH6_{400}$ 34, which was albino in appearance and displayed very stunted growth. It is likely that this strain had most or all of the *otc* cluster deleted since no antibiotic was produced on SFM or Emerson's. Spore suspensions for each putative mutant were made and used to inoculate TSB. After 48-72 hours, total DNA was isolated from harvested mycelium (**Section 2.3.2**) and analysed by PCR and Southern blotting.

4.3.5 PCR analysis of putative mutants

Recombination between the *aac(3)IV-oriT* cassette and the chromosome was confirmed by PCR (**Section 2.3.9)**. Positive controls included: pIJ773, AH6Δ*otcX3*::*aac(3)IV-oriT* and EH1Δ*otcX3*::*aac(3)IV-oriT*. Supercos1, AH6, EH1 and *S. rimosus* M4018 total DNA were used as negative controls. Analysis of the PCR products by agarose gel electrophoresis showed amplification of the *aac(3)IV-oriT* cassette in each putative mutant (**Fig 4.11a**). Amplification of *otcX3* was also conducted (**Section 2.3.8)**. Positive controls included; pET33X3, AH6, EH1 and wild-type *S. rimosus* M4018 total DNA. AH6Δ*otcX3*::*aac(3)IV-oriT* and EH1Δ*otcX3*::*aac(3)IV-oriT* were used as negative controls. Negative amplification of *otcX3* was observed from the total DNA of five putative mutants, however positive amplification was observed from six and one remained inconclusive. Putative mutants $AH6_{400}$ 28, $AH6_{400}$ 29, $EH1_{200}$ 10, $EH1_{400}$ 10 and $EH1_{400}$ 14 did not amplify *otcX3*; however, amplification of a smaller band around 800bp that did not occur in AH6/EH1, was observed (**Fig 4.11b**). The putative mutants that amplified *otcX3* all contained the *aac(3)IV-oriT* cassette, indicating that recombination may have occurred on a different section of the chromosome or that these putative mutants were possibly single crossover recombinants instead of double crossovers. Alternatively, an unforeseen genetic rearrangement may have occurred during recombination due to the unstable nature of the *S. rimosus* chromosome at the distal ends containing terminal inverted repeats that are prone to amplification and deletion and to which the *otc* cluster is located closely (Gravius *et al*., 1993; Petkovic *et al*., 2006). Weaker amplification of the 800bp fragment was also observed in these recombinants but could not be confirmed from the wild-type DNA.

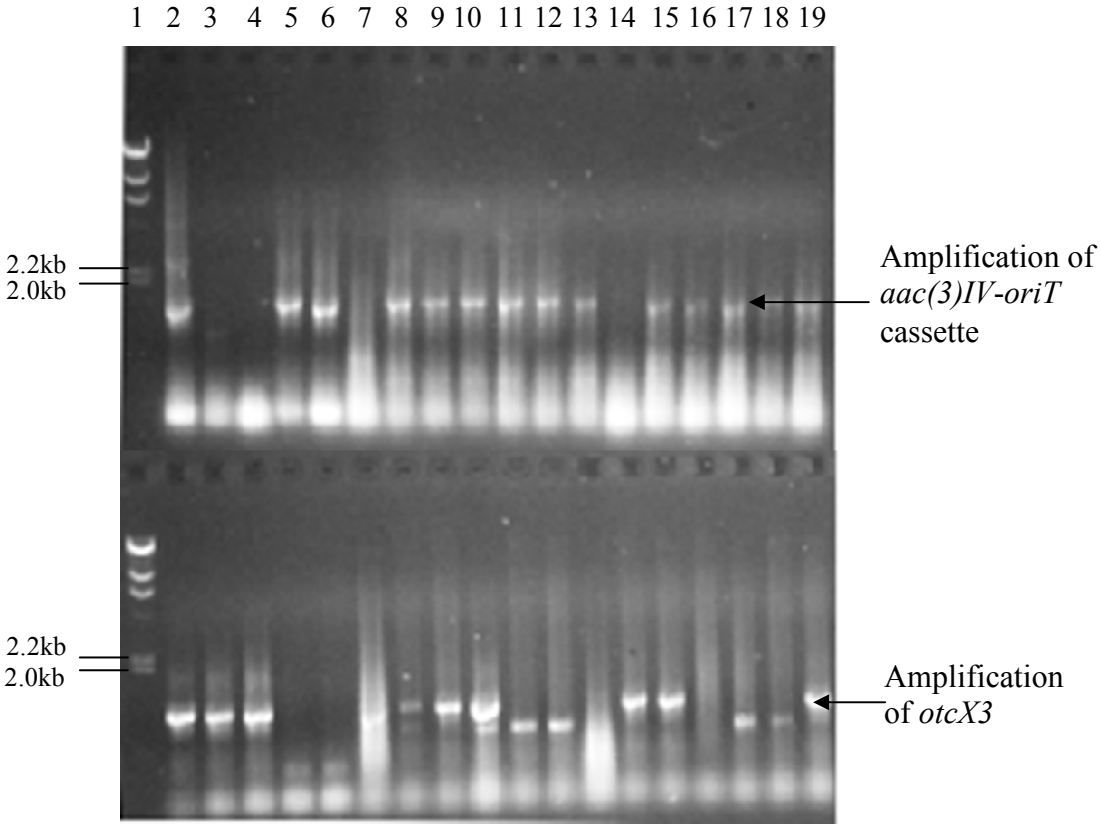


Fig 4.11a Amplification of *aac(3)IV-oriT* cassette (top) and *otcX3* (bottom) from DNA of putative mutants and *S. rimosus* M4018 wild-type

Top row: amplification of *aac(3)IV-oriT*, all lanes 5µl unless stated:
Lane 1: 125ng λ *Hin*dIII DNA marker
Lane 2: positive control amplification from pIJ773
Lane 3/4: negative controls with Supercos1/AH6
Lanes 5/6: positive controls AH6Δ*otcX3*::*aac(3)*IV-*oriT* EH1Δ*otcX3*::*aac(3)*IV-*oriT*
Lane 7: negative control amplification from *S. rimosus* M4018 wild-type total DNA
Lanes 8-13: amplification from AH6Δ*otcX3*::*aac(3)*IV-*oriT* putative mutants; AH6$_{400}$23, $_{400}$25, $_{200}$28, $_{400}$28, $_{200}$29, $_{400}$34
Lanes 14-19: EH1Δ*otcX3*::*aac(3)*IV-*oriT* putative mutants; EH1$_{400}$1, $_{200}$4, $_{200}$10, $_{400}$10, $_{400}$14, $_{400}$15
Bottom row: amplification of *otcX3*, all lanes 5µl unless stated:
Lane 1: 125ng λ *Hin*dIII DNA marker
Lane 2: positive control amplification from pET33X3
Lanes 3/4: positive control amplification from AH6/EH1
Lane 5/6: negative amplification from AH6Δ*otcX3*::*aac(3)IV-oriT*/ EH1Δ*otcX3*::*aac(3)*IV-*oriT*
Lane 7: positive control amplification from *S. rimosus* M4018 wild-type total DNA
Lanes 8-13: as top row
Lanes 14-19: as top row

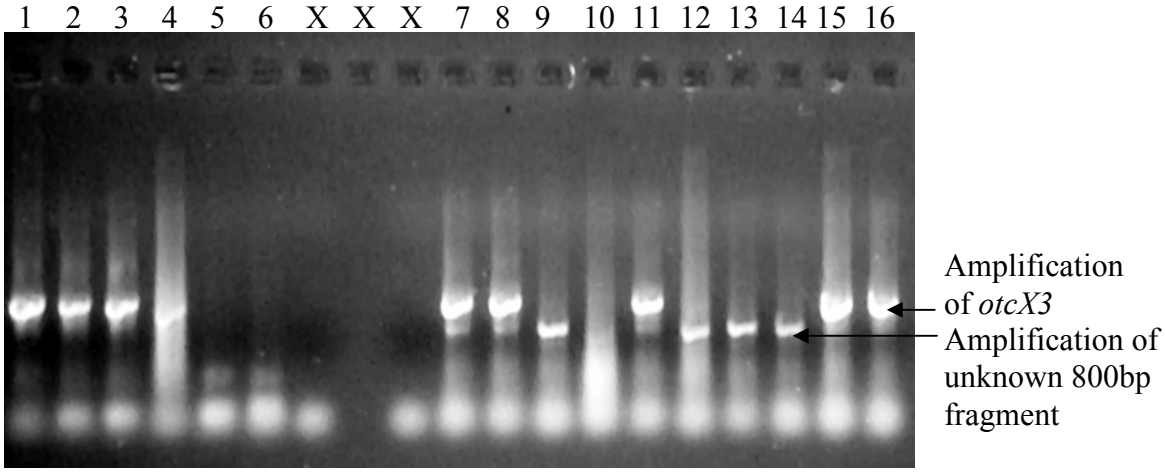


Fig 4.11b Amplification of *otcX3* from total DNA of putative mutants and *S. rimosus* M4018 wild-type

All lanes 5µl unless stated:

Lane 1: positive control using pET33X3

Lanes 2/3: positive controls amplification from AH6/EH1

Lane 4: positive control amplification from *S. rimosus* M4018 wild-type total DNA

Lanes 5/6: negative amplification from AH6Δ*otcX3*::*aac(3)IV-oriT* / EH1Δ*otcX3*::*aac(3)IV-oriT*

Lanes 7-10: amplification from AH6Δ*otcX3*::*aac(3)IV-oriT* putative mutants $AH6_{400}25$, $_{200}28$, $_{200}29$, $_{400}34$

Lanes 11-15: amplification from EH1Δ*otcX3*::*aac(3)IV-oriT* putative mutants $EH1_{200}4$, $_{200}10$, $_{400}10$, $_{400}14$, $_{400}15$

4.3.6 Southern blot analysis of putative mutants

Fig 4.12 illustrated the restriction map schema for the targeted disruption of *otcX3* on the *S. rimosus* M4018 chromosome, highlighting the relevant restriction sites. Recombination between *otcX3* and the *aac(3)IV-oriT* cassette on the plasmid would increase the size of the 5.2kb wild-type *Pst*I chromosomal fragment by 361bp to 5.5kb that would cleave to 3.45 and 2.1kb fragments upon digestion (**Section 4.3.2**). A probe of the *aac(3)IV-oriT* cassette should hybridize to the 2.1kb band alone, as the *Pst*I site is located very near the 5' end of the cassette. A probe of *otcX3* would hybridize to the wild-type 5.2kb *Pst*I fragment only, while a probe of the 5.2kb *Pst*I fragment would hybridize to both the 3.45kb and 2.1kb fragments. Probes were prepared (~1μg each) (**Section 2.3.11**). The *aac(3)IV-oriT* cassette was excised on a 1.4kb *Eco*RI-*Hin*dIII fragment from pIJ773, the 1.026kb *otcX3* excised with *Nde*I and *Bam*HI from pET33X3 and the 5.2kb *Pst*I fragment from pSRG3.

Cosmid DNA was used as a control for Southern blot analysis. Approximately equimolar band intensities were observed using 20ng cosmid DNA and 2μg total DNA. Therefore, these parameters were used to conduct the Southern blots.

Probing with the *aac(3)IV-oriT* cassette showed hybridization to the predicted 2.1kb fragment in all putative mutants, indicating that the cassette had integrated into the chromosome. The 2.1kb band was clearly visible in the mutagenized cosmids and absent in the wild-type cosmids and total DNA. However, there did appear to be some hybridization to the wild-type 5.2kb *Pst*I band, likely to have been caused by weak hybridization of the λ probe. When the DNA samples were probed with *otcX3*, hybridization to the 5.2kb *Pst*I band was observed in the wild-type cosmid and total DNA but not to the mutagenized cosmids, as expected. Hybridization to the wild-type 5.2kb fragment was found in 5 of the 11 putative mutants. A single crossover event, introducing the *aac(3)IV-oriT* cassette but retaining the wild-type sequence would explain this result. However, initial screening indicated only one isolate with resistance to both apramycin and kanamycin, the phenotype of a single crossover

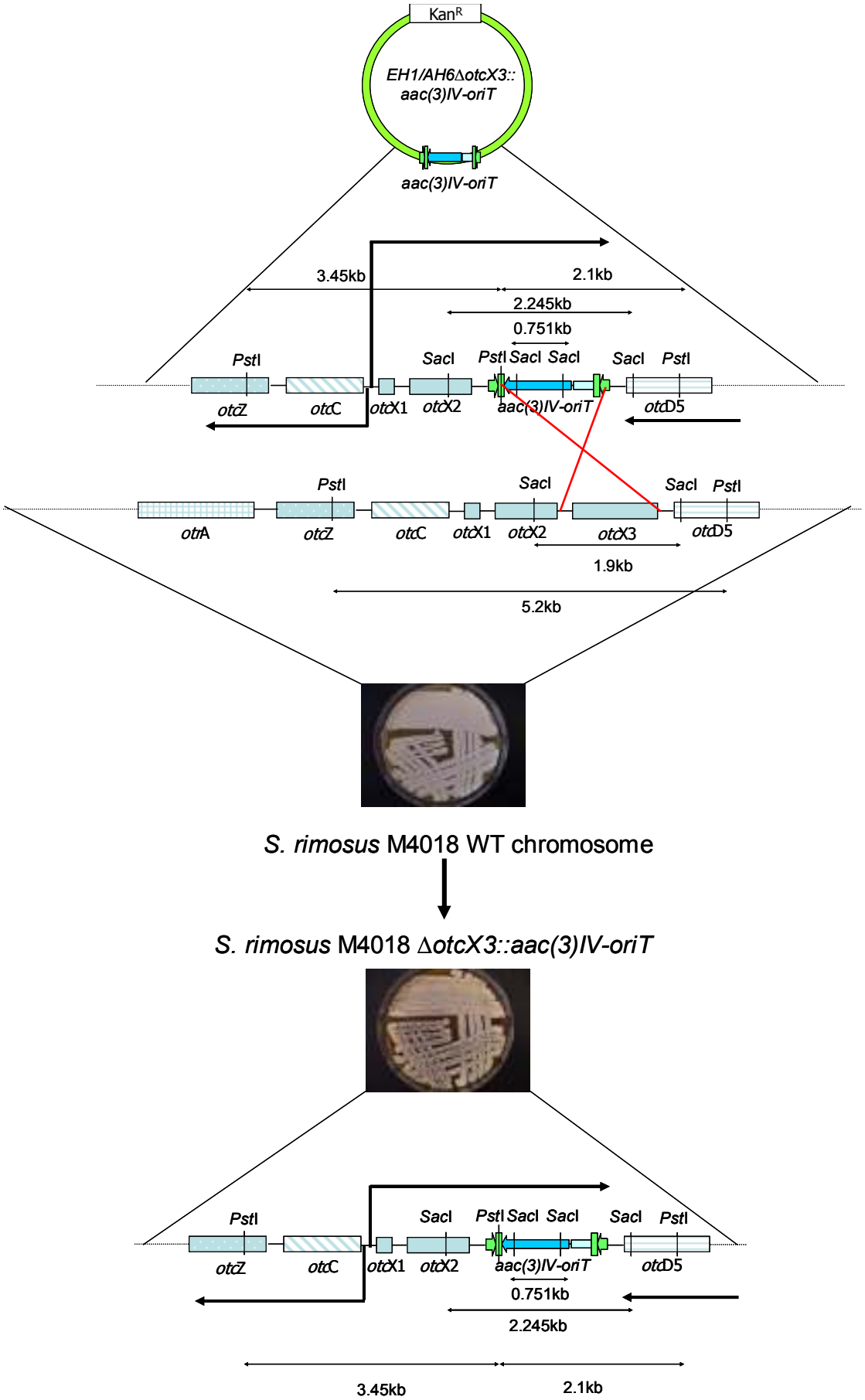


Fig 4.12 Hypothetical recombination of *aac(3)IV-oriT* with *otcX3* on *S. rimosus* M4018 chromosome
(Thicker arrows indicate direction of transcription)

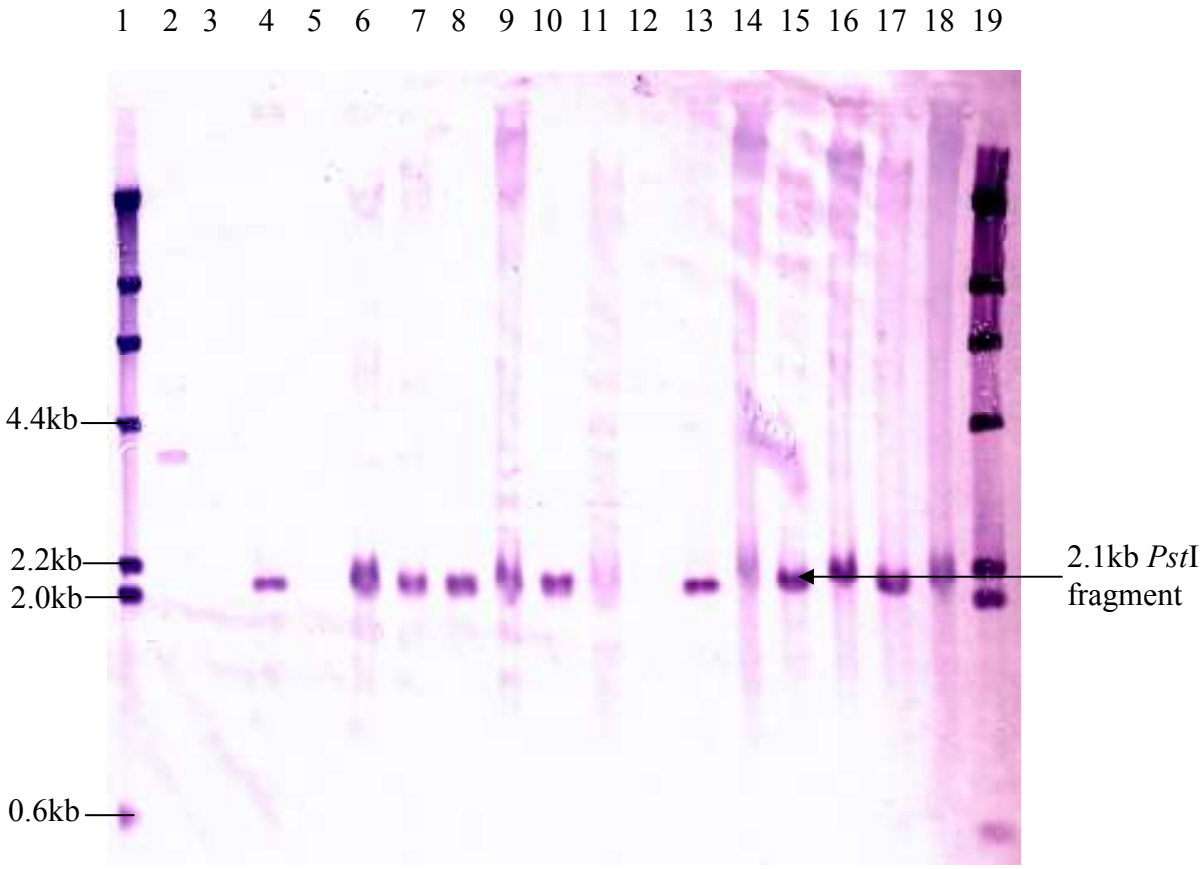


Fig 4.13 Southern hybridization of *aac(3)*IV-*oriT* probe to putative mutant DNA

Lane 1 and 19: 125ng λ *Hin*dIII marker

Lane 2: 20ng Supercos1 digested with *Pst*I

Lane 3: 20ng AH6 digested with *Pst*I

Lane 4: 20ng AH6Δ*otcX3*::*aac(3)IV-oriT* digested with *Pst*I

Lane 5: 2µg *S. rimosus* M4018 genomic DNA digested with *Pst*I

Lanes 6-11: 2µg mutants $AH6_{400}$ 23, $AH6_{400}$ 25, $AH6_{200}$ 28, $AH6_{400}$ 28, $AH6_{200}$ 29, $AH6_{400}$ 34, respectively, digested with *Pst*I

Lane 12: 20ng EH1 digested with *Pst*I

Lane 13: 20ng EH1Δ*otcX3*::*aac(3)IV-oriT* digested with *Pst*I

Lanes 14-18: 2µg mutants $EH1_{400}$ 4, $EH1_{200}$ 10, $EH1_{400}$ 10, $EH1_{400}$ 14, $EH1_{200}$ 15, respectively, digested with *Pst*I

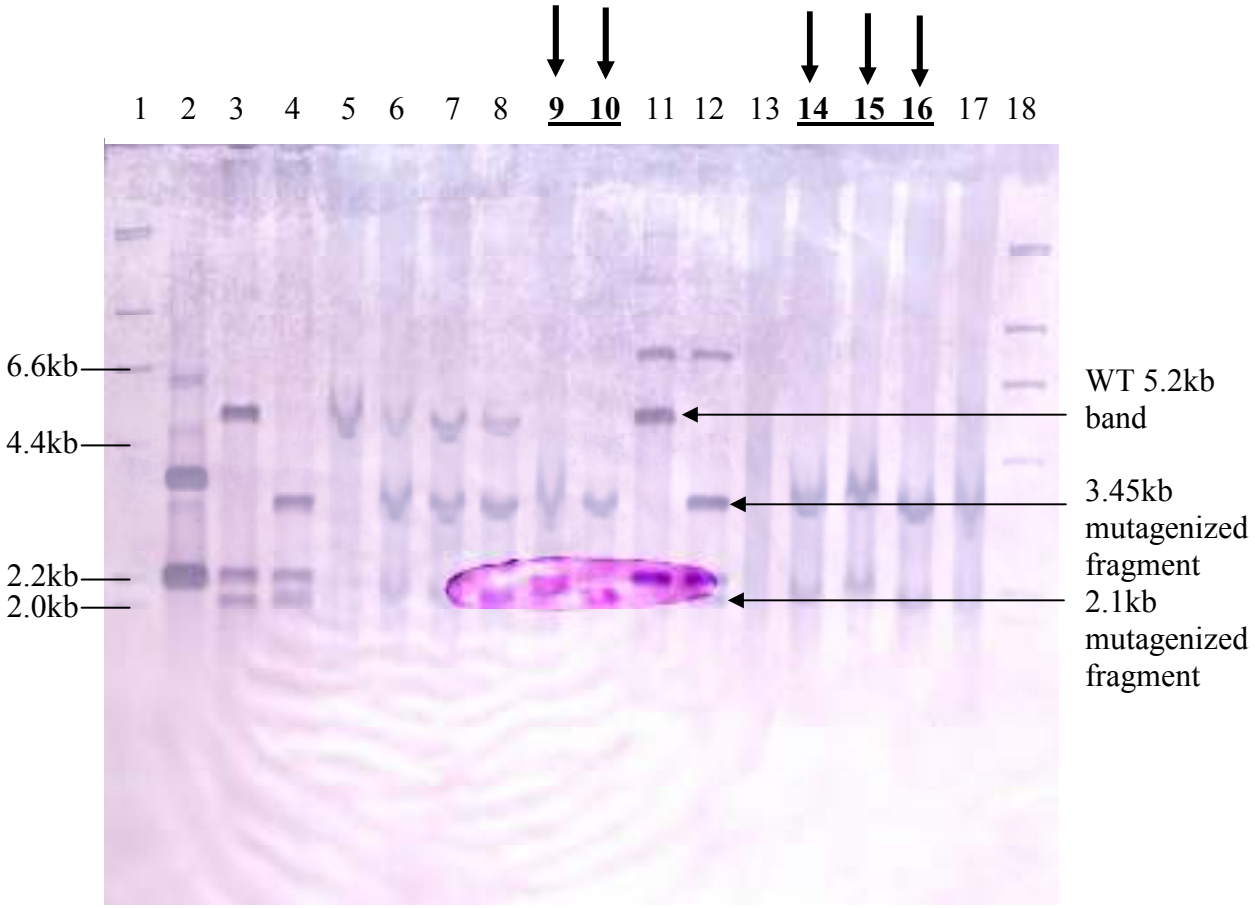


Fig 4.14 Southern hybridization of putative mutant DNA to pSRG3 probe (modified)

Lanes 1 and 18: 125ng λ *Hin*dIII marker

Lane 2: 20ng Supercos1 digested with *Pst*I

Lane 3: 20ng AH6 digested with *Pst*I

Lane 4: 20ng AH6Δ*otcX3*::*aac(3)IV-oriT* digested with *Pst*I

Lane 5: 2µg *S. rimosus* M4018 genomic DNA digested with *Pst*I

Lanes 6-10: 2µg mutants $AH6_{400}23$, $AH6_{400}25$, $AH6_{200}28$, $AH6_{400}28$, $AH6_{200}29$, respectively, digested with *Pst*I

Lane 11: 20ng EH1 digested with *Pst*I

Lane 12: 20ng EH1Δ*otcX3*::*aac(3)IV-oriT* digested with *Pst*I

Lanes 13-17: 2µg mutants $EH1_{400}$ 4, $EH1_{200}$ 10, $EH1_{400}$ 10, $EH1_{400}$ 14, $EH1_{200}$ 15, respectively, digested with *Pst*I.

event (**Fig 4.10**); however it was not selected for further analysis. Probing with the wild-type 5.2kb *Pst*I fragment produced the blot in **Fig 4.14**. The probe hybridized to the mutagenized 3.45kb and 2.1kb *Pst*I fragments, as expected. Control hybridization to the corresponding wild-type sequence on the cosmids AH6 and EH1 and the wild-type total DNA was also confirmed. The 'double-hybridization' result observed from probing with *otcX3* was confirmed by hybridization to the 5.2kb fragment in the same putative mutants and was also observed in a further three; $AH6_{200}$ 28, EH1 4 and EH1 15. Five of the putative mutants (**Fig 4.14)** had a hybridization pattern consistent with the hypothesized allelic exchange, and were chosen for further characterization.

The 'double' hybridization could have been due to partially-digested total DNA, which may have produced 3.45/2.1kb fragments as well as the undigested 5.5kb fragment. However, amplification of *otcX3* from total DNA isolated from the same putative mutants in **Fig 4.11** contradicted this. This 'double' hybridization was caused by the formation of a wild-type-mutant hybrid during recombination, producing a 'heterokaryon' (Sermonti *et al*., 1960). In this situation, recombinants would have contained a copy of the wild-type and mutagenized chromosomes, which would therefore explain the amplification of *otcX3* from PCR (**Fig 4.11**) and hybridization to both wild-type and mutagenized fragments in Southern blot analysis (**Fig 4.14**).

A promoter for the *otcX* locus, *otcXp1*, was previously identified (McDowall *et al*., 1991), located between *otcC* and *otcX1* (**Fig 4.12**). The promoter that drives the 'leftwards' transcription of *otcC*, *otcZ* and *otrA* does so as a polycistronic mRNA (Doyle *et al*., 1991). Transcription from the *otcX* promoter is driven 'rightwards', transcribing *otcX1*, *otcX2* and terminating at *otcX3*, so the transcription of *otcX1* and *otcX2* should not be affected by the absence of *otcX3*, hypothetically. *OtcD5* lies immediately to the right of *otcX3* but is transcribed 'leftwards' by the promoter starting at *otcD1* (**Fig 1.9**). Disruption of *otcX3* should therefore have no effect on the transcription of *otcD1*.

A correlation between pigmentation and OTC production in *S. rimosus* was documented by Gravius *et al*., (1993). The putative mutants displayed a similar phenotype to the wild-type on Emerson's agar and SFM, which indicated that the mutagenized *otc* cluster was still functional. The morphologies of the selected mutants were compared to the wild-type on Emerson's agar and SFM under apramycin selection, as well as without. It was clear that, on both media, the mutants were naturally darker than the wild-type. Either more of, or a darker pigment, was being produced under the extra stress of antibiotic selection.

The putative mutants of EH1 that also displayed the correct allelic exchange in the Southern blots ((2)10, (4)10 and 14) all had the same phenotype on TSA, Emerson's Agar and SFM. Those strains that would appear most likely to be 'heterokaryons' instead of single crossovers (4 and 15) showed a slightly darker brown phenotype on TSA. Likewise, the AH6 putative mutants showing this same pattern (23, 25 and (2)28) showed a darker phenotype than the wild-type. It will be important to characterize the metabolites produced by each putative mutant.

4.3.7 Attempted complementation of putative mutants

An attempt was made to complement the mutants disrupted at *otcX3* by reintroducing the wild-type gene into the putative mutant using a *Streptomyces* vector. The *otcX3* gene was excised from the *E. coli* expression vector pET33X3 (**Section 2.1.2**), as a 1.026kb *Nde*I-*Bam*HI fragment and ligated into a *Streptomyces* expression plasmid, pATet2 (Tilley, personal comm.); a modified version of the *Streptomyces* integration vector pSET152 (Bierman *et al*., 1992). pATet2 was created by the addition of the thiostrepton resistance gene on a 1.1kb *Eco*RI fragment and the 350bp *erm*E* (Gramajo *et al*., 1991) constitutive promoter to drive the expression of any insert located downstream. This also contained the sequence for the ribosomal binding site, tuf1 (Vijgenboom *et al*., 1994).

pATet2 contained an insert on an *Nde*I/*Bam*HI fragment under control by the *erm*E*tuf1 promoter. This was excised followed by ligation of the 1.026kb *Nde*I/*Bam*HI *otcX3* insert into the open vector to create pATX3 (**Appendix A. 12, Section 2.1.2**). The ligation mixture was then used to transform *E. coli* JM109 (**Section 2.1.1**) and transformants were selected with apramycin. The plasmid was then isolated and used to transform *dam*$^{-}$ *dcm*$^{-}$ *E. coli* ET12567. The *otcX3* insert was excised from pATX3 and the 5' overhangs from restriction digestion were blunt-ended using dNTP's and Klenow enzyme. The blunt-ended vector was then self-ligated to form pAT3, which was subsequently used to transform *E. coli* JM109 and further transformation of *E. coli* ET12567. pAT3 was created to provide a negative control for complementation with pATX3.

Cultures of the putative mutants were prepared for electroporation (**Section 2.2.14**, Pigac and Schrempf, 1995). Transformants were selected with thiostrepton at 10μg.ml^{-1} and apramycin at 400μg.ml^{-1} on Oxoid TSA. After 48 hours, thiostrepton-resistant transformants were streaked on to SFM to make spore suspensions of each strain (**Section 2.2.5**). It was assumed that pATX3 would integrate into the chromosome at the *attB* site on the chromosome of the putative mutant, thus expressing the *otcX3* cassette from the *erm*E* promoter and returning any novel metabolic phenotype to the wild-type status.

4.3.7.1 Hybridization of wild-type 5.2kb *Pst*I fragment to DNA from putative mutant strains transformed with pATX3

Total DNA was isolated from cultures of the thiostrepton-resistant strains, and probed with the 5.2kb *Pst*I fragment containing *otcX3*. Upon successful integration of pATX3 into the chromosome of EH1$_{14}$ at the *attB* site, the new sequence would introduce three new *Pst*I sites (**Fig 4.15)**. Digestion with *Pst*I would therefore cleave a 4.5kb fragment containing the *otcX3* insert that the 5.2kb fragment would hybridize to. The results from hybridization of the 5.2kb *Pst*I fragment to the DNA of the transformed putative mutants digested with *Pst*I were shown in **Fig 4.16.**

Probing with the 5.2kb fragment did not display hybridization to the hypothesized 4.5kb *Pst*I fragment, nor any other new fragments that were not previously observed in **Fig 4.16**. However, a positive control using digested pATX3 was not included, flawing the experimental interpretation. PCR was used to test for the presence of *otcX3* in the thiostrepton-resistant strains (**Fig 4.17**). Amplification of *otcX3* from pATX3 was confirmed from the positive controls and absent in the negative control from pATet2. The strains transformed with pATX3, did not amplify *otcX3*. The $EH1_{14}$ mutant showed amplification of the 800bp fragment observed in the earlier PCR analysis and weak amplification of a fragment approximately 1kb in size. This same result was then observed from the DNA of the transformed mutants indicating that *otcX3* had not been re-introduced. Therefore, despite conferring resistance to thiostrepton, no evidence of the integration of pATX3 into the chromosome could be confirmed.

It was strange that this band was only amplified in the disrupted mutants and not the wild-type. It was clear that the *aac(3)IV-oriT* cassette did not recombine with the *otcX3* insert from pATX3 as all the transformed mutants displayed the same restriction pattern as the original mutant. The intensity of the PCR product of *otcX3* from the *S. rimosus* genomic wild-type template or any of the other positive controls was not reproduced in any of the mutants. The 'transformed' mutants were able to grow healthily under thiostrepton selection, yet no further evidence could be found to confirm that complementation of the original mutation had been achieved. Another Southern blot, using a thiostrepton probe may confirm the presence of the thiotrepton resistance gene but a more logical route would be to adopt a new strategy to compliment the disruption of *otcX3*.

4.3.8 Hybridization of DNA from mutant strains to *otrB* probe

As discussed in **Section 1.3**, genetic instability is highly prevalent in *S. rimosus* due to 550kb tandem inverted repeats at either end of the linear chromosome that are prone to amplification and subsequent deletion (Gravius *et al*., 1993). The *otrB* gene

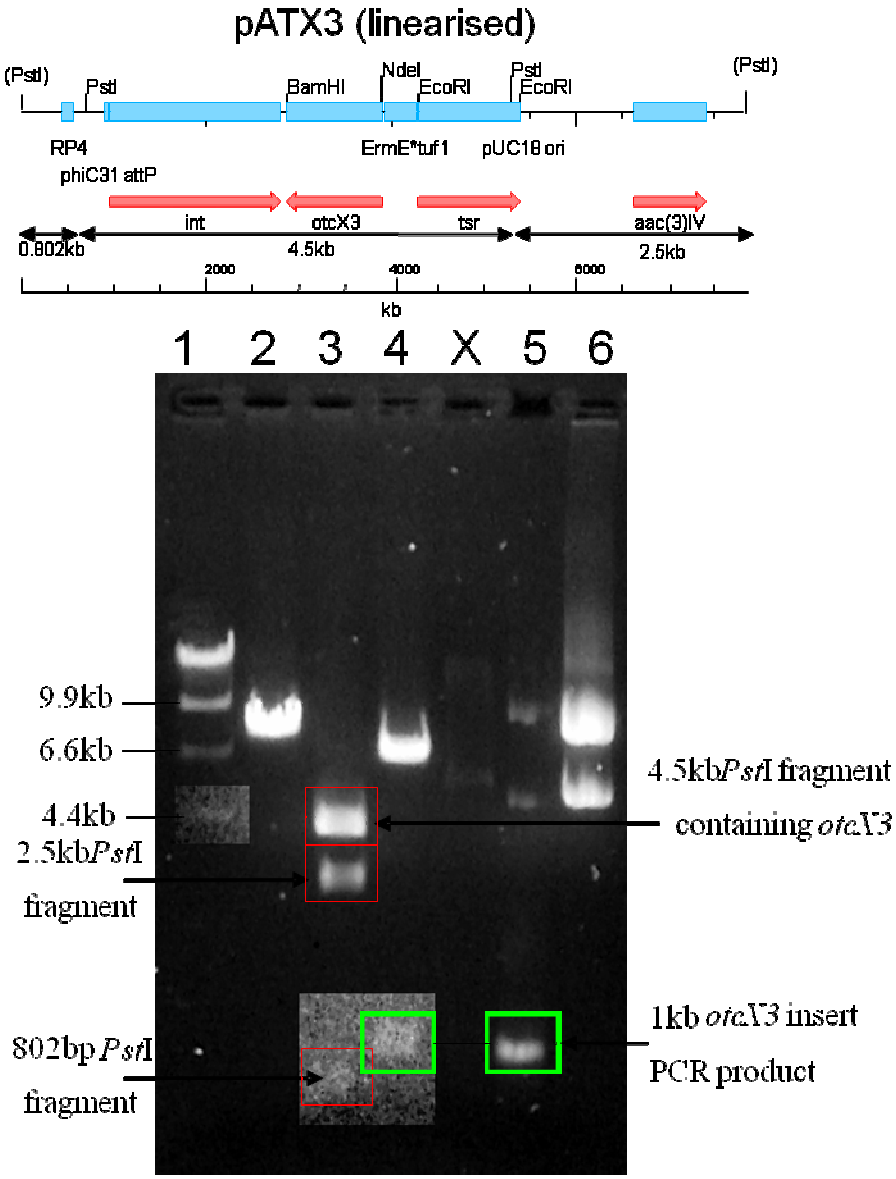


Fig 4.15 Restriction analysis of pATX3

Lane 1: 125ng λ *Hin*dIII marker

Lane 2: 100ng pATX3 linearised with *Bam*HI

Lane 3: 100ng pATX3 digested with *Pst*I

Lane 4: 100ng pATX3 digested with *Nde*I/*Bam*HI

Lane 5: 50ng PCR product from amplification of *otcX3*

Lane 6: 100ng uncut pATX3

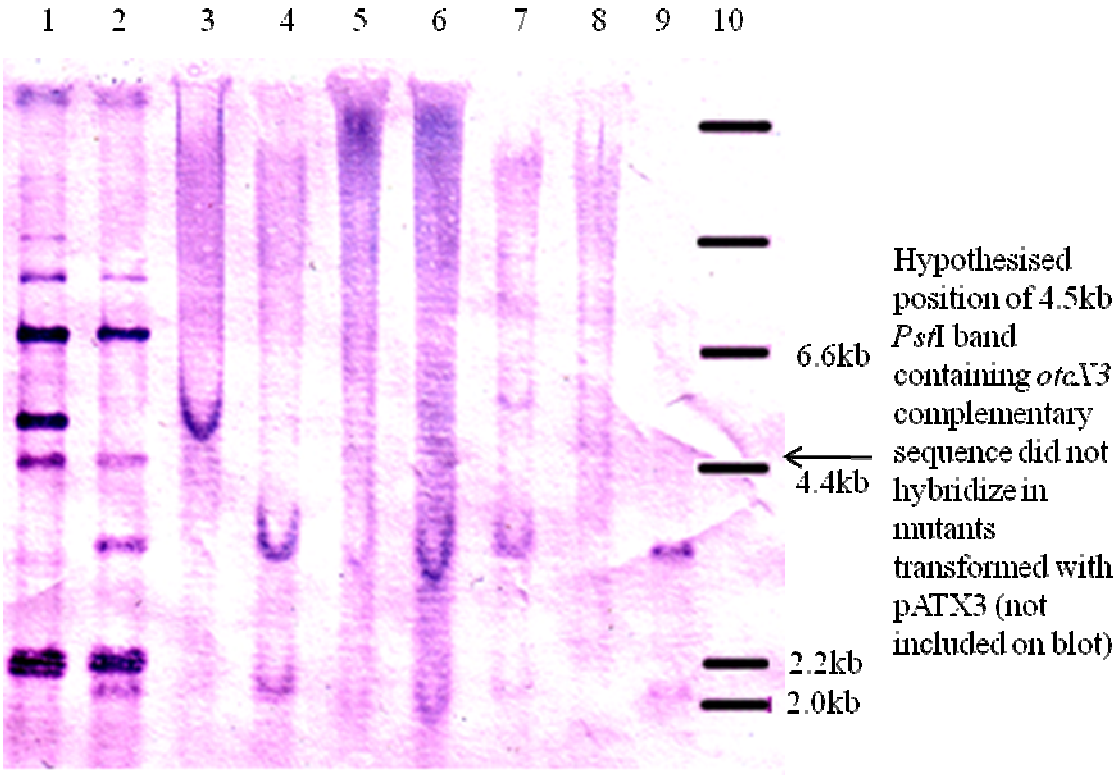


Fig 4.16 Southern hybridization of mutant DNA to wild-type 5.2kb *Pst*I probe

Lane 1: 20ng EH1 digested with *Pst*I

Lane 2: 20ng EH1Δ*otcX3*::*aac(3)IV-oriT* digested with *Pst*I

Lane 3: 2µg total DNA from *S. rimosus* M4018 digested with *Pst*I

Lane 4: 2µg total DNA from putative mutant $EH1_{14}$ digested with *Pst*I

Lanes 5-8: 2µg total DNA from $EH1_{14}$ putative mutants transformed with pATX3 (2, 3, 6, 8, respectively) digested with *Pst*I

Lane 9: total DNA from 2µg $EH1_{14}$ putative mutant transformed with pAT3

Lane 10: λ *Hin*dIII marker (drawn in using Adobe Photoshop)

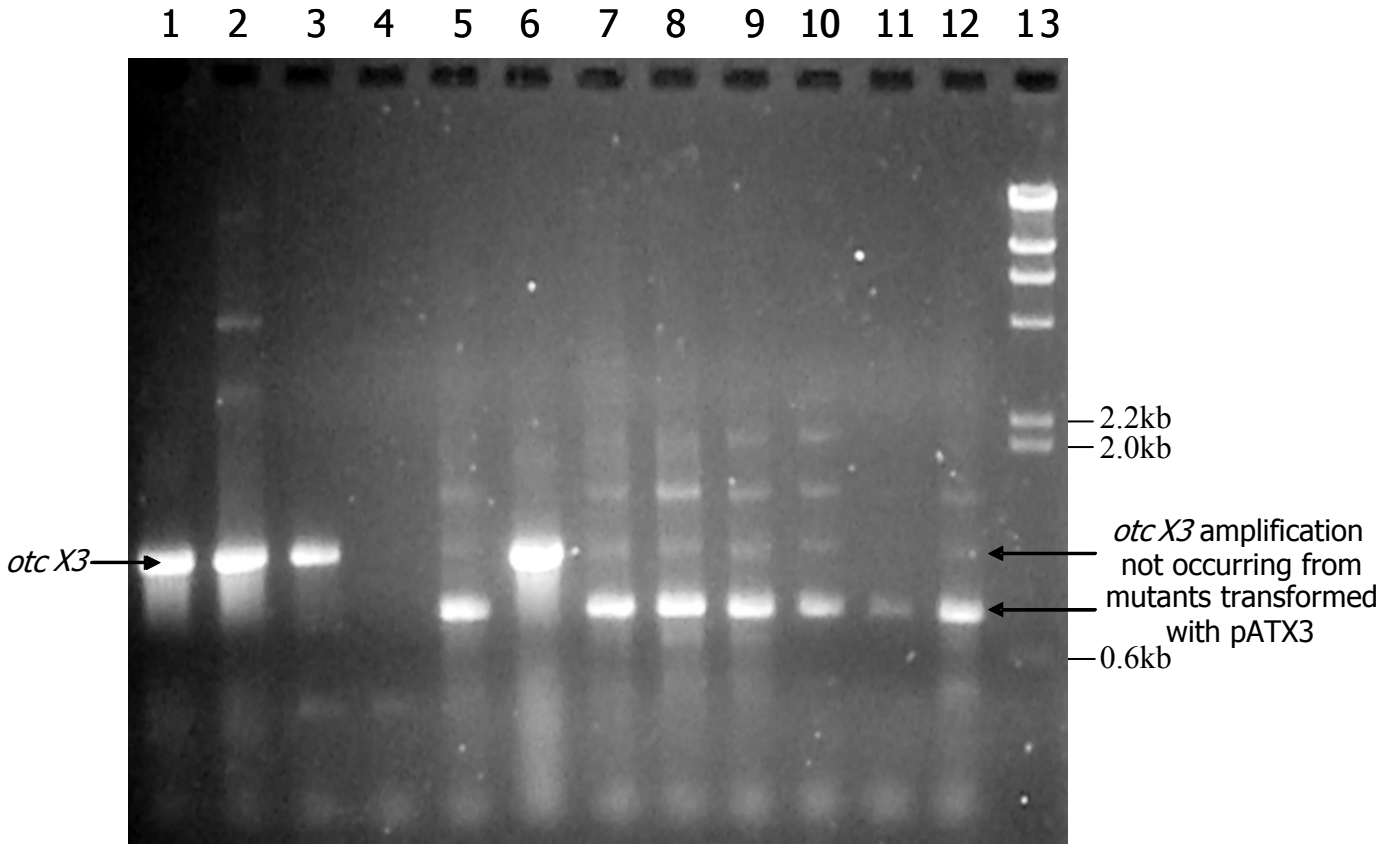


Fig 4.17 Unsuccessful amplification of *otcX3* from total DNA of mutants transformed with pATX3

Lane 1: positive control amplification from EH1

Lane 2: positive control amplification from pET33X3

Lane 3: amplification of *otcX3* from pATX3

Lane 4: negative amplification of *otcX3* from pATet2

Lane 5: negative amplification from from $EH1_{14}$ *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* putative mutant

Lane 6: positive control amplification from *S. rimosus* M4018 wild-type

Lanes 7-12: negative amplification of *otcX3* from putative mutant $EH1_{14}$ transformed with pATX3

Lane 13: 125ng λ *Hin*dIII marker

represents the terminal ORF in the *otc* cluster and the gene nearest the repeat region. Therefore, the *otrB* region deletes most frequently. It is located on a 2.6kb *Bam*HI/*Sac*I fragment (**Fig 1.9**). This fragment was used to probe (**Section 2.3.14**) total DNA of both the wild-type *S. rimosus* M4018 and putative mutants (**Fig 4.18**). The cosmids EH1 and EH1Δ*otcX3*::*aac(3)IV-oriT* were used as positive controls for the 2.6kb *Bam*HI/*Sac*I fragment and the probe hybridized to both. The probe also hybridized to the same fragment in the wild-type, the putative mutant and the strains transformed with pATX3. In lanes 4-6, containing wild-type DNA, DNA from the mutant and one of the strains transformed with pATX3, respectively, hybridization was observed to bands at around 3kb and 4.4kb that were not seen in the rest of the mutants, nor the cosmid controls.

Analysis of the map of the *otc* cluster (**Fig 1.9**) revealed no further *Bam*HI/*Sac*I fragments of these sizes. Therefore, this suggests that these bands were caused by partial digestion created by *Bam*HI/*Sac*I digestion of DNA out with the cluster.

4.3.9 Characterization of putative mutant strains using agar-plug bioassays

Agar-plug bioassays were used to test the bioactivity of the metabolites produced by putative mutants and those transformed with pATX3. Two pairs of isogenic *E. coli* strains were used for the experiment and bioassays were assembled (**Section 2.5)**. Of each pair, one strain was tet-sensitive (strains AB2834, AB2848) and the other tet-resistant (AB2834::Tn*10*, AB2848::Tn*10*, **Section 2.1.1**). The plasmid pBR322, encoding TetA(C) tetracycline resistance (McNicholas *et al*., 1992; Chopra *et al*., 1992, **Appendix A. 13**), was used to transform each of the four strains of *E. coli* to act as a control when testing for sensitivity to tetracycline.

Fresh 25ml SFM plates of *S. rimosus* M4018, several strains of Δ*otcX3*::*aac(3)IV-oriT* putative mutants transformed with pATX3 and one Δ*otcX3*::*aac(3)IV-oriT* putative mutant strain not transformed with pATX3 were prepared ($EH1_{14}$), inoculated simultaneously and grown to maturity over several days. Antibiotic

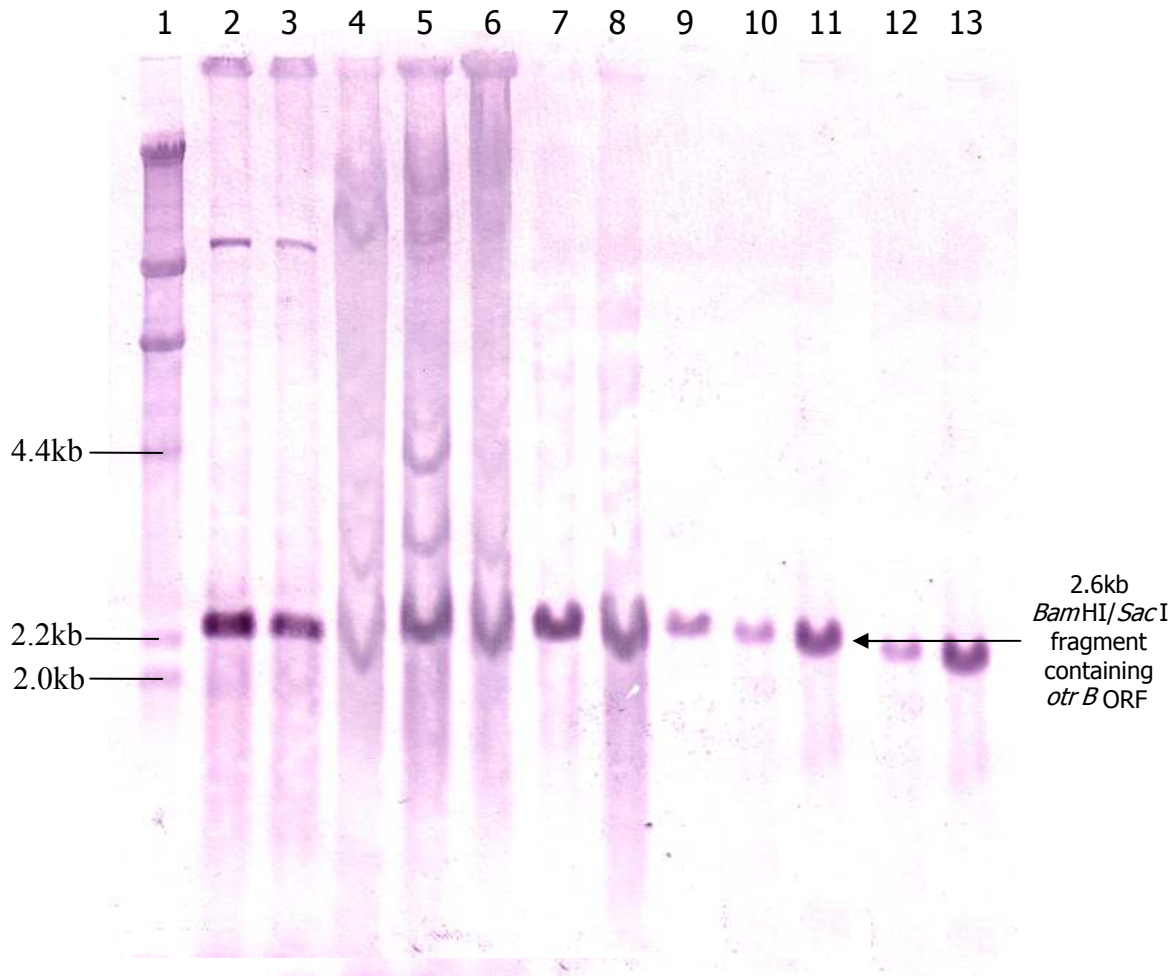


Fig 4.18 Southern hybridization of mutant DNA to *otrB* probe

Lane 1: 25ng λ *Hin*dIII marker

Lane 2: 20ng EH1 digested with *Bam*HI/*Sac*I

Lane 3: 20ng EH1Δ*otcX3*::*aac(3)IV-oriT* digested with *Bam*HI/*Sac*I

Lane 4: 2µg *S. rimosus* M4018 genomic DNA digested with *Bam*HI/*Sac*I

Lane 5: 2µg $EH1_{14}$ mutant digested with *Bam*HI/*Sac*I

Lanes 6-11: 2µg $EH1_{14}$ mutants transformed with pATX3 (2, 3, 6, 8, 10, respectively) digested with *Bam*HI/*Sac*I

Lanes 11-12: 2µg $EH1_{14}$ mutants transformed with pAT3

selection using apramycin was not used to prevent the need for an apramycin control in the test medium. Transformation of the putative mutant strains with the control plasmid pAT3 had not yet been successful when this experiment was conducted and therefore could not be used.

Against the tetracycline-sensitive *E. coli*, the *S. rimosus* M4018 wild-type and the L-agar plug control containing tetracycline at a concentration of 20μg.ml^{-1} (referred to as Tc^{20} herein) zones of inhibition were produced, as expected. However, a large zone of inhibition was observed around the agar-plug of the putative mutant ($EH1_{14}$), indicating that it was still able to synthesis OTC. Those mutants that had been transformed with pATX3 also displayed even larger zones of inhibition. When the same tetracycline-sensitive *E. coli* were transformed with pBR322 no clear zones were observed around any of the agar plugs (**Fig 4.19**).

The larger zones of inhibition around the putative mutants transformed with pATX3 indicated that a higher concentration of bioactive metabolite was synthesised by these strains. It could not be concluded however, that a novel phenotype had been generated in the Δ*otcX3*::*aac(3)IV-oriT* putative mutant. Also, despite being resistant to thiostrepton, the strains transformed with pATX3 had not confirmed the hypothesized integration of the construct when probed with pSRG3 containing *otcX3*, therefore complementation to the disruption of *otcX3* could not be verified. The agar-plug bioassay was repeated with three other strains of *E. coli* containing Tn*10*-mediated tetracycline resistance; *E. coli* DS883, ET12567 and DS9614. Again, no clear zones were observed, in accordance with the results from the initial assay.

The agar-plug bioassay was repeated against Gram-positive bacteria (**Fig 4.20**). To conduct this this, various strains of multi-drug resistant methicillin-resistant *Staphylococcus aureus* (MRSA) were used. Four strains of MRSA were obtained, initially (Smith, K. personal comm.), of which two were previously characterized as tetracycline-sensitive and two tetracycline-resistant. Of these strains, M11, also

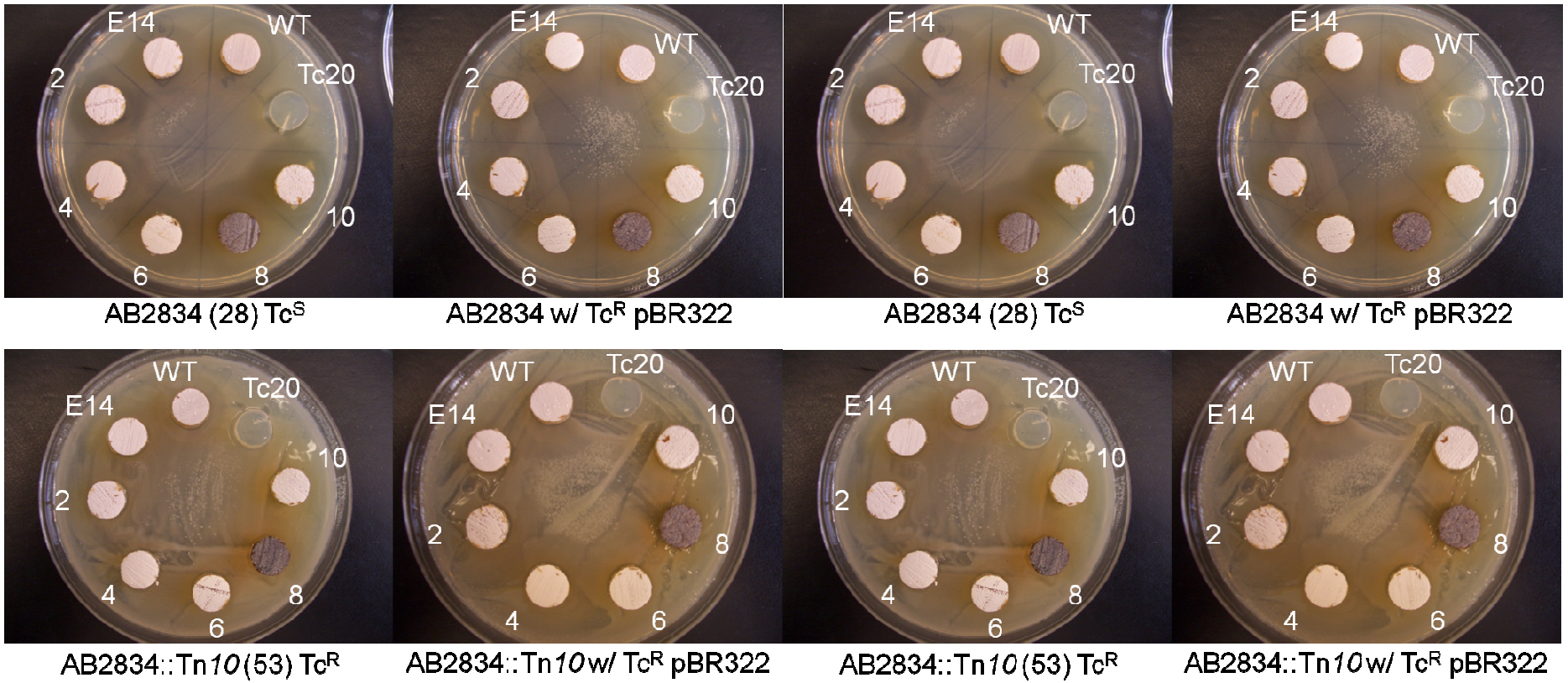


Fig 4.19 **Agar-plug bioassay of *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutant transformed with pATX3 against Tc^S (28, 29) and Tc^R (53, 54) strains of isogenic *E. coli***

Key: Tc20 - Tetracycline LA plug at 20µg.ml^{-1}; WT - *S. rimosus* M4018 WT; E14 = *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* putative mutant; 2, 4, 6, 8, 10 = putative mutants transformed with pATX3

carried resistance genes for 14 antibiotics used clinically, not including tetracyclines and methicillin.

The metabolites produced by the putative mutant strains were active against the tetracycline-sensitive strains of MRSA, M3 and M13. M3 and M13 were sensitive to the wild-type and Tc^{20} agar-plug controls as well as the metabolites from each mutant strain. The tetracycline-resistant strains, M2 and M11, were resistant to the Tc^{20} and wild-type controls. M2 was also resistant to the metabolites produced by the putative mutant and the strains transformed with pATX3. However, M11, resistant to 16 clinical antibiotics, was sensitive to the metabolites of the mutants and the wild-type strain. Since both M2 and M11 displayed resistance to the Tc^{20} control plugs, it could be concluded that resistance to tetracycline was being expressed in both strains. It was postulated from these results that the clear zones observed around the putative mutant strains were a result of different mechanisms of tetracycline resistance, with the unknown mechanism within M11 encoding a lower minimum inhibitory concentration in comparison to other strains. To investigate this further, the bioassay was repeated using more strains of tetracycline-resistant MRSA; M7, M12, M25, M31 and M52 (Smith, K. personal comm.). In this instance, tetracycline and oxytetracycline controls were used at a final concentration of $40\mu g.ml^{-1}$ (Tc^{40}/OTC^{40}).

Of these strains (including M2 and M11), four displayed phenotypic resistance to the Tc^{40} and OTC^{40} control plugs as well as the metabolites produced by the *Streptomyces* strains; M2, M7, M12 and M25. The other three strains; M11, M31 and M52, displayed very small zones of inhibition against the Tc^{40} and OTC^{40} control plugs (these were difficult to observe on the photographs but could be seen on closer examination by the human eye) and were sensitive to the metabolites produced by the *Streptomyces* strains.

M11, M31 and M52 showed varying degrees of sensitivity to the metabolites produced by the *Streptomyces* strains, but the smallest zone of inhibition on each

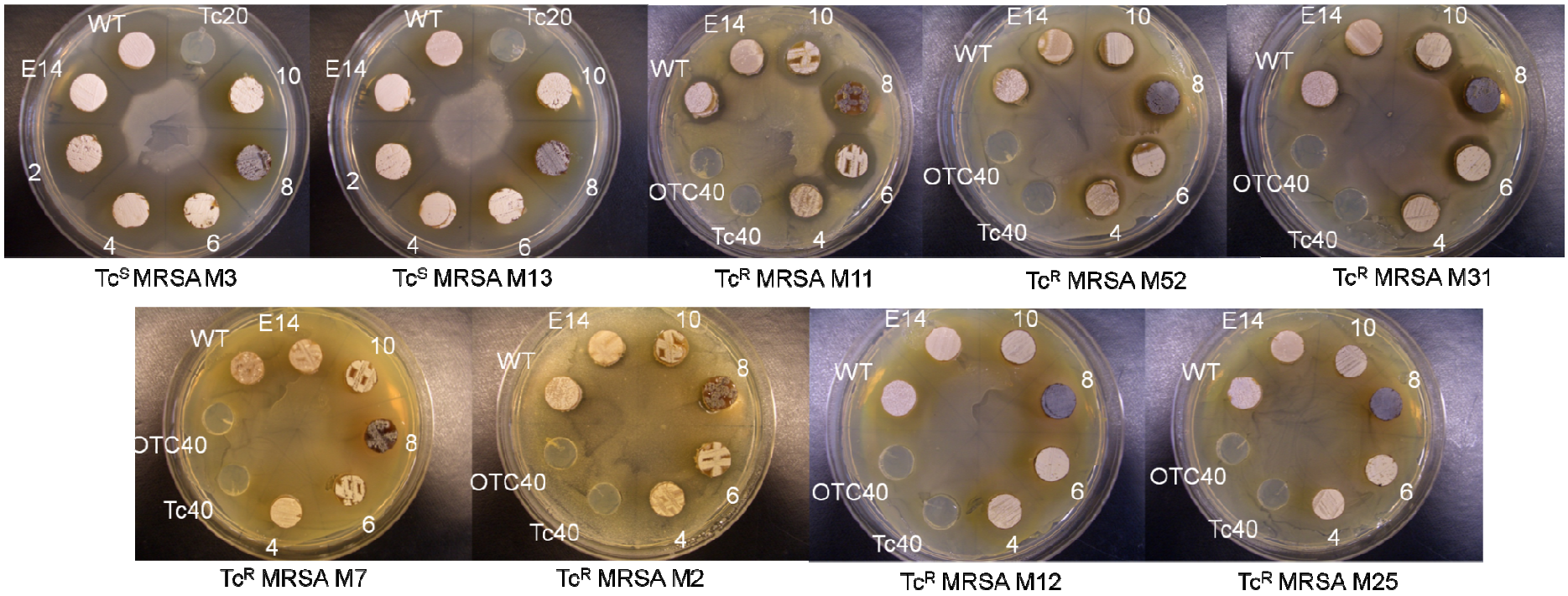


Fig 4.20 **Agar-plug bioassay of *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* putative mutant transformed with pATX3 against Tc^S (M3, M13) and (Tc^R) (M11, M52, M31, M7, M2, M12, M25) MRSA**

Key: Tc20/40 - Tetracycline LA plug at 20/40µg.ml^{-1}; OTC40 - Oxyetracycline LA plug at 20/40µg.ml^{-1}; WT - *S. rimosus* M4018 WT; E14 - *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* putative mutant; 2, 4, 6, 8, 10 = mutants transformed with pATX3

plate was observed around the $EH1_{14}$ putative mutant strain and the largest around strain no. 8. This result reinforced the initial observation of M11 that these strains (M11, M31, M52) encoded mechanisms of tetracycline resistance that conferred lower minimum inhibitory levels of resistance to tetracyclines than the mechanism encoded by the strains that displayed complete resistance to the controls and *Streptomyces* strains (M7, M2, M12, M25, **Fig 4.20**) . Unfortunately, no clarification of the specific genotype of each strain was available to confirm these phenotypes.

4.3.10 TLC and HPLC analyses of metabolites produced by putative mutants

To follow up the results from the agar-plug bioassays, data on the chemistry of the bioactive compounds produced by the putative mutants was required. Therefore, TLC analysis was conducted, as described in **Section 2.6**. No clear difference in the RF values of the spots was observed between the metabolic extracts of the *S. rimosus* M4018 wild-type, the putative mutant or the mutants transformed with pATX3.

Separation of the metabolic extracts was thus repeated. The plate was dried and placed in a large, sterile petri-dish. L-agar, inoculated with a 1/100 concentration of an overnight culture of Tc^S *E. coli*, was poured over the plate before allowing it to set. The plate was then incubated overnight at 30^{o}C. Clear zones created by diffusion of the antibiotics from the TLC plate to the surface of the agar were subsequently observed (**Fig 4.21**). The mean diameter of each spot was taken as the migration point for the antibiotics. This distance, divided by the migration distance of the mobile phase solvent was used to calculate RF values for each sample.

RF values were calculated at 0.13, 0.134 and 0.138 for the OTC standard, *S. rimosus* M4018 wild-type and Δ*otcX3*::*aac(3)IV-oriT* putative mutant strains, respectively. This result indicated that the chemical structure of the bioactive metabolite from the mutants was similar to OTC, thus reinforcing the observation from agar-plug bioassays that the putative mutant was still able to produce OTC. To clarify this, HPLC analysis of the metabolic extracts was conducted.

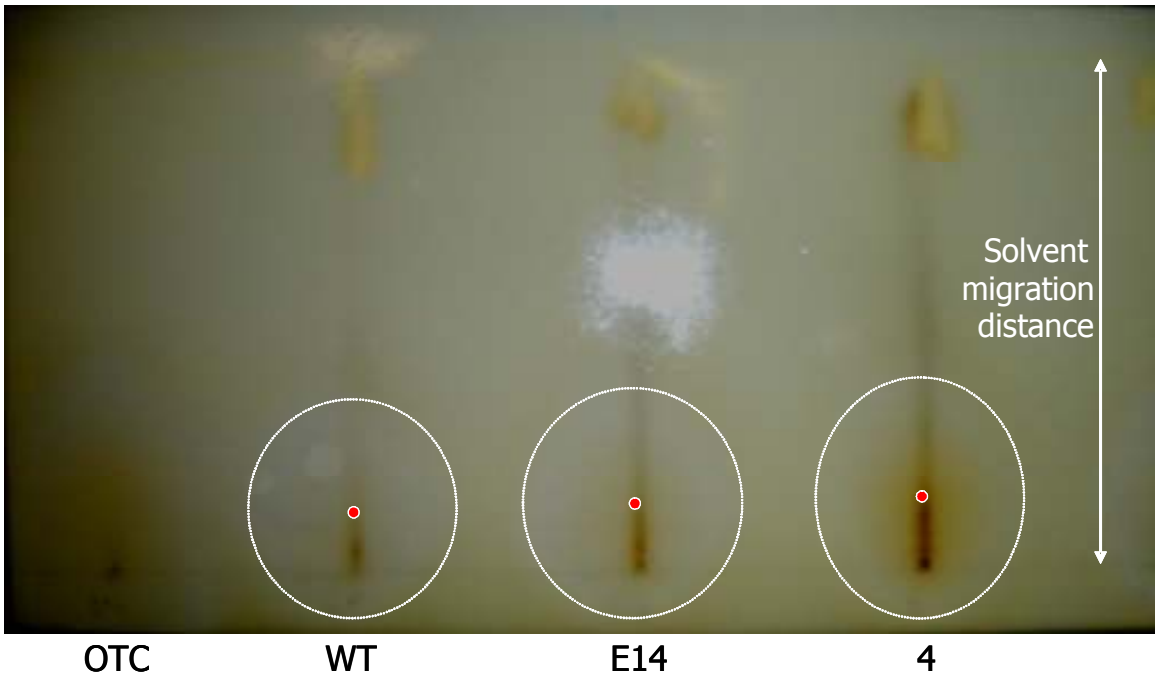


Fig 4.21 Bioassay of mutant extract TLC plate against Tc^S *E. coli*

Key:

OTC = oxytetracycline standard (150µg)

WT = *S. rimosus* M4018 wild-type extract

E14 = *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutant

4 = mutant transformed with pATX3

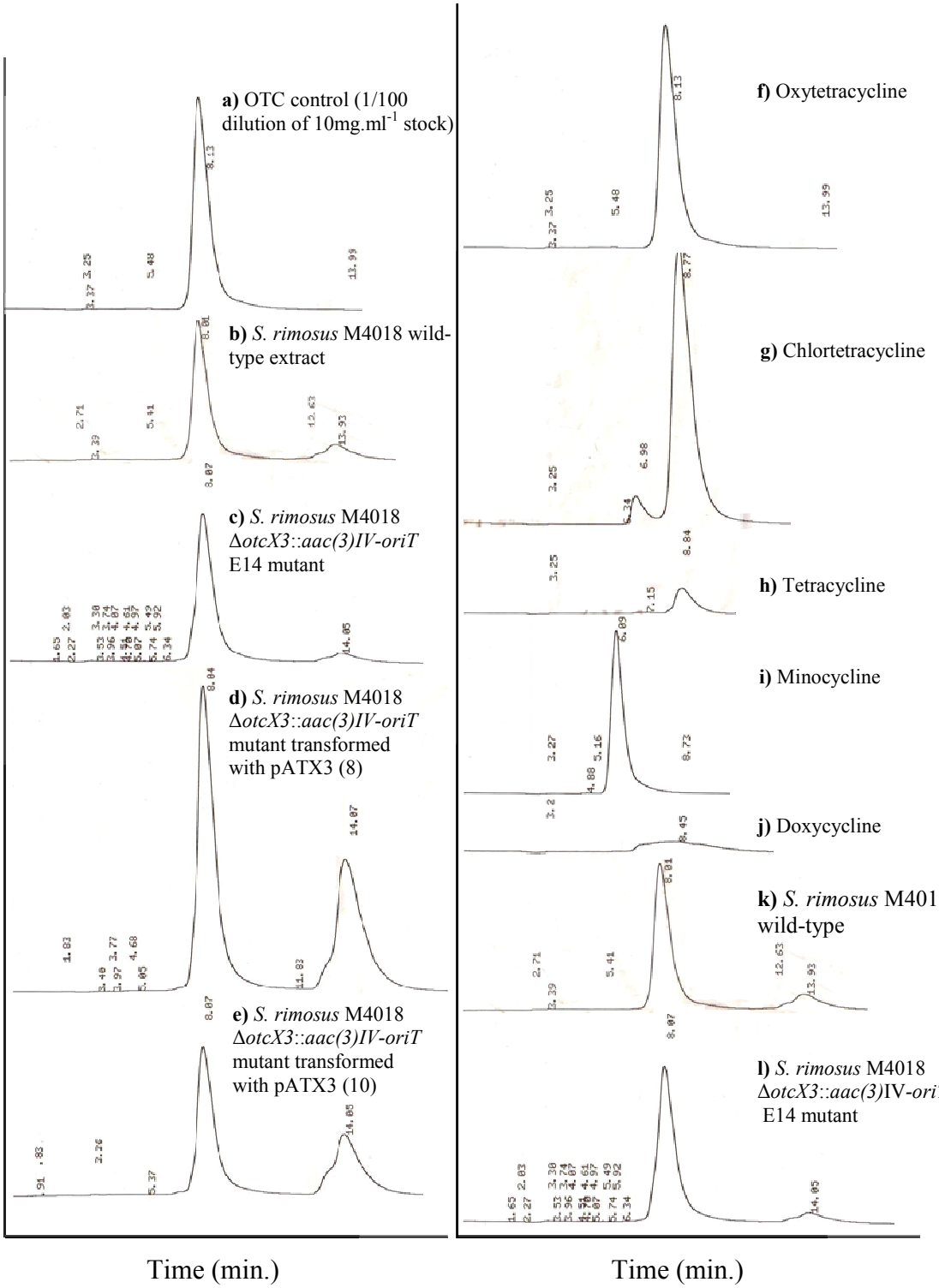


Fig 4.22 Chromatograms of *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutant extract HPLC analysis

An HPLC experimental setup was assembled, as described in **Section 2.7**. In this case, a 1/100 dilution of a 10mg.ml^{-1} OTC standard solution was injected on to the column as the control with the attenuation set to 256 on the integrator and 0.5% transmittance on the UV-Vis detector, itself set to a wavelength of 350nm. The filtered extracts, solubilised with the mobile phase were then also injected on to the column and the retention time (RT) for each sample was recorded by the integrator. These data were summarised and aligned in **Fig 4.22(a-e)**. For further control comparisons, 1/100 dilutions of 10mg.ml^{-1} stock solutions of other tetracyclines were also injected onto the column in addition to OTC; minocycline, doxycycline, chlortetracycline and tetracycline. These were aligned for comparison with OTC, the wild-type and the putative mutant in **Fig 4.22(f-l)**.

The chromatograms in **Fig 4.22** concurred with the observations from agar-plug bioassays that the putative mutant was still able to synthesise OTC. Thus, the PCR-targeted disruption of *otcX3* had not abolished OTC production. The chromatograms of the *S. rimosus* M4018 wild-type and the mutant showed peaks at an almost identical RT to the OTC control. The RT of the OTC control was 8.17 minutes, while the RT of the peaks in the wild-type and the mutant were 8.13 and 8.07 minutes, respectively. The RT of the strains transformed with pATX3, 8 (8.04 min) and 10 (8.07 min) were also very similar.

A combination of two smaller peaks was also observed with the wild-type and mutant extracts. In the wild-type and putative mutant the second of the two peaks showed an RT of 14.12 and 14.05 minutes, respectively, while mutants 8 (14.07 min) and 10 (14.05 min) displayed larger peaks indicating a production of these compounds at a much higher concentration. The retention times as well as the peak shapes produced by tetracycline (8.84 min), chlortetracycline (8.77 min), doxycycline (8.45 min) and minocycline (6.09 min) were clearly distinguishable from the second OTC control (8.13 min), thus reinforcing the evidence that the putative mutant could still synthesise OTC. Ideally, ADOT would have been used as a control for this analysis; however, none was available at the time.

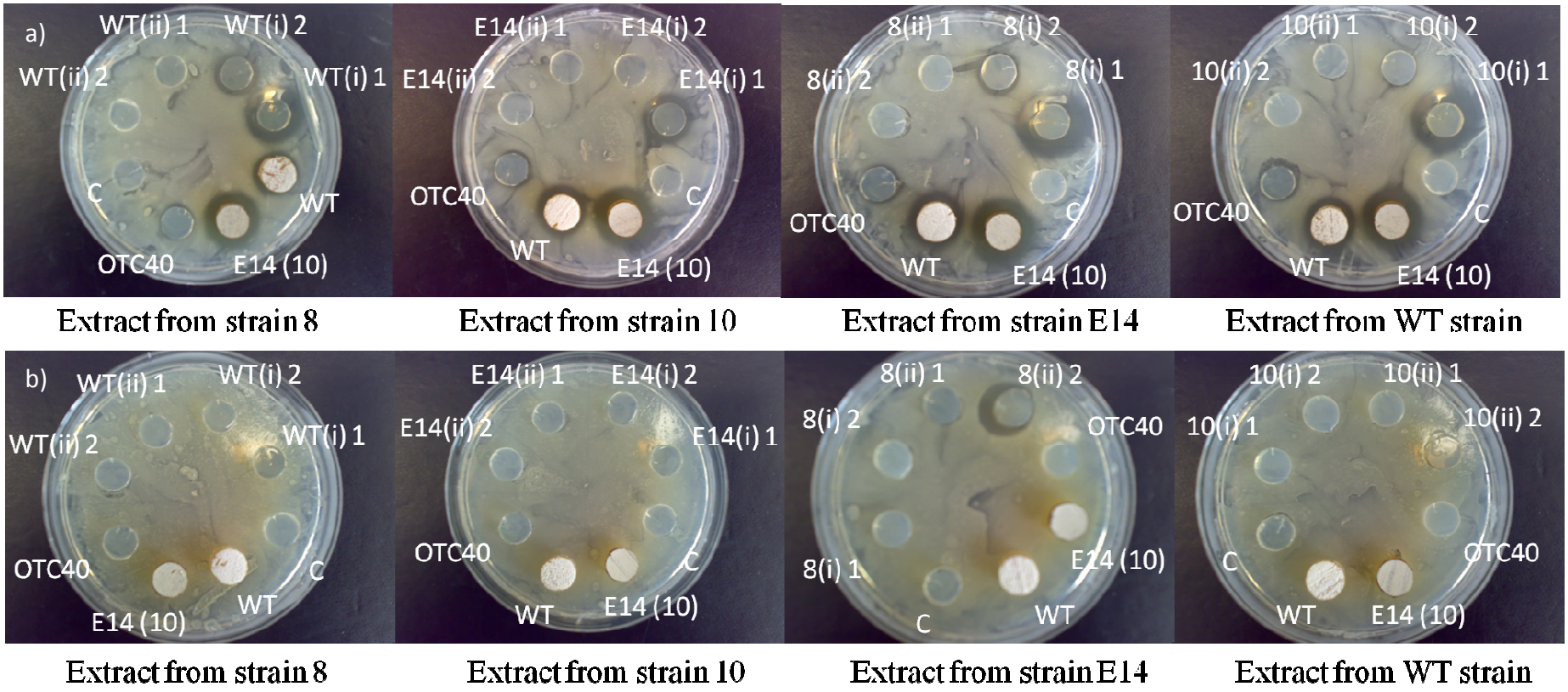


Fig 4.23 Agar-plug bioassay of HPLC fractions against a) MRSA strain 11 and b) tetracycline-resistant MRSA strain

Key: OTC 40 = Oxytetracycline LA plug at 40µg.ml^{-1}; WT = *S. rimosus* M4018 wild-type; E14 = *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutant; 10 = *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutant transformed with pATX3; Pk1(i)/(ii) / Pk2(i)/(ii) = 1st/2nd part of fraction collected from first/second peak of HPLC elution (Fig 4.25); C = Fraction collected from HPLC elution out with the peaks at 350nm (Fig 4.25).
* clear zone created by ethanol spill when adding samples to agar plugs.

The strains that produced the largest zones of inhibition against the bacteria tested in the bioassays (8 and 10) also displayed higher concentrations of the compounds in the secondary peaks. To investigate whether the larger zones of inhibition could be attributed to the presence of these compounds at a higher concentration compared to the wild-type and putative mutant, fresh concentrated samples of each extract were prepared. After injecting these on to the column, the fractions for the peak presumed to contain OTC and the two secondary peaks were collected separately, along with a sample of the HPLC elute from which no peaks were observed as a control and concentrated to dryness overnight under vacuum evaporation at room temperature. The bioactivity of these fractions were then tested by agar-plug bioassays. The MRSA strains M7 and M11, showing resistance and sensitivity, respectively, in the bioassay (**Fig 4.20**) were used as the test strains. The *S. rimosus* M4018 wild-type and strain 10, along with a plug supplemented with 40μg.ml^{-1} OTC were used as controls. The evaporated fractions were resuspended in 20μl absolute ethanol, added to the agar plugs and allowed to dry. After overnight incubation at 37^{o}C, the plates were photographed and presented in **Fig 4.23**.

No sensitivity of MRSA strain M7 to any of the HPLC elutions was visible, as expected. This concurred with the results in **Fig 4.20**, in which these tetracycline-resistant strains of MRSA displayed complete resistance to the *S. rimosus* M4018 wild-type and *S. rimosus* M4018 Δ*otcX3*::*aac(3)IV-oriT* mutants.. The result in **Fig 4.23** confirmed that the compounds from the secondary peaks were not bioactive. Clear zones were observed around the plugs containing the concentrated fractions from the first major peak of UV absorbance at 350nm but no clear zones formed around the plugs containing the fractions from the secondary peaks. No clear zones were observed around the control plug, therefore it could be concluded that the bioactive compound was exclusive to the compound from the primary peak, presumed to be OTC in accordance with the data collected.

4.4 Conclusions

- The REdirect protocol was successfully adapted for *S. rimosus* M4018 using electroporation instead of conjugation to introduce mutagenic DNA (**Section 4.1-4.2**). However, for future reference, selection of apramycin-resistant transformants should always be conducted using a minimum final concentration of 400μg.ml^{-1} on TSA.
- Disruption of *otcX3* on cosmids containing the *otc* cluster was successful in *E. coli*, as shown by PCR and restriction analysis. After introduction of mutagenic cosmid DNA into the *S. rimosus* wild-type, phenotypic analysis of transformants indicated double crossover events in every isolate and only one instance of a single crossover event.
- Southern blot analysis indicated that the *aac(3)IV-oriT* cassette had integrated into the *otc* cluster at the correct position, thus disrupting *otcX3*; however, some strains showed evidence of both wild-type and mutagenized DNA present in the same strain. Putative mutants that displayed only mutagenic allelic exchange were selected for characterization.
- Transformation of selected putative mutants with pATX3 produced thiostrepton-resistant colonies but integration could not be confirmed by either PCR or Southern blot analysis, therefore complementation of *otcX3* disruption could not be confirmed and remains incomplete.
- Characterization by agar-plug bioassays against *E. coli* and MRSA indicated that OTC biosynthesis was retained in the putative mutant strains despite disruption of *otcX3*. TLC and HPLC analysis confirmed this, thus the hypothesis that functional *otcX3* is essential for OTC biosynthesis was contradicted by the data accumulated. Therefore, the function of *otcX3* remains unclear.

Chapter 5:
Discussion

From the results achieved in this work, it can be concluded that the protocol for PCR-targeted mutagenesis (Datsenko and Wanner, 2000) was successfully adapted for *S. rimosus* M4018. Media composition was shown to be critical for identification of putative mutants. TSA, a medium that represses OTC production at the transcriptional level due to a high level of phosphate (McDowall *et al*., 1999), was the only available medium that provided adequate growth conditions for selection with apramycin.

Characterization of metabolic extracts from the Δ*otcX3*::*aac(3)IV-oriT* putative mutant indicated that replacement of *otcX3* was a non-polar event and did not abolish OTC biosynthesis. Therefore, no new pharmacological phenotype was discovered, thus disproving the hypothesis that *otcX3* was essential for the incorporation of the malonamyl starter unit. Therefore, the role of *otcX3* remains unclear. Transformation of the putative mutant with pATX3, which was designed to complement the loss of functional *otcX3,* produced thiostrepton-resistant strains but integration of the phage vector into the chromosome could not be confirmed by PCR or Southern blot analysis when probed with the 5.2kb wild-type fragment containing *otcX3*.

Characterization of the metabolite(s) produced by the mutant using agar-plug bioassays revealed interesting results. The hypothesis that OTC biosynthesis would be abolished and replaced by biosynthesis of ADOT, or a compound with very limited antimicrobial properties in the absence of functional *otcX3*, was disproved. The putative mutant produced a compound(s) that displayed similar antimicrobial properties to the wild-type against tetracycline-sensitive *E. coli*. The bioactivity of the metabolites produced by the mutant was also tested against Gram-positive bacteria using various strains of MRSA (Smith, K. personal comm.). Each strain conferred resistance to multiple antibiotics in addition to methicillin. Zones of inhibition were observed around agar-plugs of the tetracycline-sensitive strains, as seen with the tetracycline-sensitive *E. coli*, thus indicating that the putative mutant was still able to synthesise an antibiotic with broad-spectrum activity, likely to be OTC.

The bioassay was repeated using more 'tetracycline-resistant' strains of MRSA. Of seven strains, four were completely resistant and three were sensitive to the metabolites of the *Streptomyces* strains, thus illustrating that the latter (M11, 52, 31) had a lower tolerance to Tc/OTC, presumably due to a different mode of tetracycline resistance (**Fig 4.20)**.

After TLC analysis confirmed that the putative mutant was producing a compound with a similar RF to the wild-type, HPLC analysis (**Fig 4.22-4.23**) of the metabolic extract of the mutants also revealed a major peak of UV absorbance at 350nm at the same RT and shape of peak as the OTC control. This same peak was also found in the extract of the *S. rimosus* M4018 wild-type. Various samples of other known tetracyclines; tetracycline, chlortetracycline, minocycline and doxycycline were also injected on to the column. These compounds all produced peaks of different shapes and with noticeably different retention times. Ideally, ADOT would have been used as a control during the TLC and HPLC analyses to conform fully to the hypothesis; however, none was available at the time. Secondary peaks were also detected in the extract of the *Streptomyces* strains at a later RT at 350nm. Analysis of fractions from both peaks showed that only the first peak contained a compound that was bioactive against MRSA. Therefore, it could be concluded that the mutant was able to synthesise OTC.

Zhang *et al*., (2006) conducted co-expression studies of *otc* biosynthetic genes based on the model provided by the expression construct pRM5 for studies of the *act* biosynthetic genes in the heterologous host, *S. coelicolor* CH999 (McDaniel *et al*., 1993) as part of an extensive study to elucidate the essential genes required for OTC biosynthesis (**Section 1.7**). As an extension of the system described by McDaniel *et al*., (1993), co-expression of the *otc* minimal PKS genes *otcY1-1 – 1-3* along with the C-9 ketoreductase of the *act* cluster, *actIII*, produced isomers of the compound RM20(b,c) (**Fig 5.1c**), as observed with co-expression of the tetracenomycin minimal PKS and *actIII* (McDaniel *et al*., 1993; Fu *et al*., 1994b). This showed that the *otc* minimal PKS was able to synthesise acetate as well as malonamate-derived

polyketides, and concurred with the discovery of ADOT from metabolic extracts of earlier uncharacterized mutants of *S. aureofaciens* and *S. rimosus* (Hochstein *et al*., 1960; Fu *et al*., 1994b).

As a putative acyltransferase exclusive to the *otc* cluster, *otcX3* was hypothesised to be crucial for the incorporation of malonamyl. However, *otcX3* was found not to be one of the essential genes required to synthesise a compound containing an amide group. The minimal PKS genes, *otcY1-1 – otcY1-3* and the amidotransferase gene, *otcY1-4* (creating pWJ85), were the minimum genes required to produce a novel compound containing an amide group, WJ85 (**Fig 5.1a**), which confirmed the incorporation of the malonamyl starter unit (Zhang *et al*., 2006b). Additional co-expression of the C-9 ketoreductase, *otcY2-1* (pWJ35), led to the production of the alkaloid WJ35 as the major product (**Fig 5.1b**), while removal of *otcY1-4* abolished biosynthesis of WJ35. Furthermore, no increased incorporation of malonamyl-CoA was observed when *otcX3* was co-expressed in addition to the genes required for WJ35 synthesis (Zhang *et al*., 2006). The structures of WJ85 and WJ35 proved that *otcY1-4* was the essential gene for biosynthesis and incorporation of malonamyl into the OTC polyketide backbone. Consequently, the true function of *otcX3* remains unclear.

H^1 NMR analysis of WJ85 and WJ35 indicated that the aromatic rings observed in WJ85 were created by spontaneous cyclisation of the 19C polyketide backbone since no catalytic regioselectivity could account for the structure of the molecule (Zhang *et al*., 2006b). On the contrary, the structure of the 19C WJ35 showed that the regioselectivity of ketoreduction at C-9 of the nascent polyketide backbone by OtcY2-1 was responsible for the difference in structure between WJ85 and WJ35. This was confirmed when *otcY2-1* was replaced by its homologue in ACT biosynthesis, *actIII* (Fernandez-Moreno *et al*., 1992). WJ35 was still synthesised *in vivo*, thus confirming relaxed substrate specificity in each enzyme (Zhang *et al*., 2006).

Co-expression of a heterologous minimal PKS (*act*, Zawada and Khosla, 1997 and *tcm,* Shen and Hutchison, 1993) with *otcY1-4* and *otcY2-1* did not produce any amidated compounds. Previous studies had shown that co-expression of these genes with the ACP encoded by *otcY1-3* could produce novel compounds derived from acetate and malonyl subunits (Khosla *et al*., 1993). However, this data indicated that the *otc* minimal KS/CLF is unique in its ability to accept acetyl and malonamyl-derived priming units compared to its *act*/*tcm* homologues (Zhang *et al*., 2006).

Removal of *otcY1-4* from pWJ83 (to form pWJ83c) produced desmethylaklanoic acid (**Fig 5.1d**), the structure of which showed the correct cyclisation of rings B, C and D yet ring A was incomplete. The structure of desmethylaklanoic acid showed that no malonamyl unit had been incorporated, thus reinforcing the evidence for the critical role of *otcY1-4* but also indicating how important the presence of the C2 amide group, provided by incorporation of malonamyl, was for the formation of the final ring (ring A) of OTC (Zhang *et al*., 2007). It was hypothesized that formation of ring A could occur spontaneously, probably as a result of dissociation of the cyclised intermediate from the phosphopantetheine arm of the ACP (Zhang *et al*., 2007).

This data proved that *otcY1-4*, and not *otcX3*, was the essential gene required for the assembly and incorporation of malonamyl into the OTC backbone. *OtcY1-4* was subsequently assigned as part of an 'extended' minimal PKS for OTC biosynthesis. While the essential function of *otcY1-4* is clear, the data published by Zhang and co-workers could not discount an alternative role for OtcY1-4 later during assembly of OTC biosynthesis. In this hypothesis, the minimal PKS would be primed by a malonyl group and chain elongation would proceed as normal. The polyketide chain would subsequently be amidated by OtcY1-4 to yield the compound WJ35. However, it would be difficult to determine the timing or the substrate of this reaction (Zhang *et al*., 2006). A model for the hypothesized biosynthesis of malonamyl-CoA/ACP by an energy-dependent OtcY1-4-catalysed transfer of the amide group of a glutamine residue to a malonyl subunit is illustrated in **Fig 5.2**.

No putative acyltransferase gene is found in the chlortetracycline (CTC) gene cluster of *S. aureofaciens* (Ryan *et al*., 1994; I.S. Hunter, personal comm.), which is surprising considering that both compounds contain the carboxamido group. It is possible that a homologue of *otcX3* exists outside the *ctc* gene cluster, elsewhere on the chromosome; however, when the *ctc* cluster was cloned and introduced into *S. lividans* CTC biosynthesis was confirmed, implying that the *ctc* cluster contains the essential genetic information required (Ryan *et al*., 1999). This indicates that no cluster-specific acyltransferase or homologue of *otcX3* is essential for CTC biosynthesis, which is consistent with the data published by Zhang *et al*., (2006) and observations made in this work that *otcX3* is not essential for OTC biosynthesis.

The core of essential genes for ATC biosynthesis from genes in the *otc* cluster was established and discussed previously, identifying the minimal PKS (*otcY1-1 - 1-4*), C-9 ketoreductase (*otcY2-1*), first ring cyclase (*otcD1*), C-6 methylase (*otcY2-5*), oxygenase (*otcD2*), SAM-dependent transaminase (*otcX2*) and *otcZ* (see detailed discussion in **Section 1.7**, Zhang *et al*., 2008). Combined with *otcC* (Peric-Concha *et al*., 2005) and the putative gene(s) required for the final conversion of 5-DHOTC to OTC (Nakano *et al*., 2004; Zhang *et al*., 2006) these represent the essential genes required for OTC biosynthesis. Following this, metabolic analysis of an *otcY1-5* mutant was the first example of a 'non-essential' gene to be characterized from the *otc* cluster (Wang *et al*., 2009).

It was revealed that *otcY1-5* functions as an 'assistant' oxygenase to its 'essential' partner, *otcD2*. Broad-range HPLC and NMR analysis of extracts produced by the *otcY1-5* mutant identified the shunt product, PW1 (**Fig 5.1e**) as well as a ~50% lower yield of OTC (Wang *et al*., 2009). An *otcD2* mutant also produced the shunt product, PW2 (**Fig 5.1f**) and removal of *otcY1-5* from the plasmid pWJATC4 produced JX11 (**Fig 5.1g**) after co-expression in *S. coelicolor* CH999. Analysis of these compounds confirmed the role of OtcY1-5 as an ancillary oxygenase that works in tandem with OtcD2 during the hydroxylation of 6-MPT to 4-hydroxy-MPT and thus, minimises the production of shunt products from the pathway (**Section 1.7**, Wang *et al*., 2009).

This suggests that broad-spectrum HPLC analysis of the extracts of the Δ*otcX3::aac(3)IV-oriT* mutant could possibly reveal further shunt product(s) that could characterize the function of *otcX3*.

The proteins DpsC and DpsD, hypothesized to be fundamental for the incorporation of the propionyl starter unit in the biosynthesis of daunorubicin/doxorubicin from *S. peucetius* were studied *in vitro* and it was found that only DpsC was required for the incorporation of the propionyl starter unit into the polyketide backbone (Bao *et al*., 1999). In addition, disruption of the *otcX3* homologue *dpsD*, did not abolish daunorubicin biosynthesis (Rajgarhia *et al*., 2001). While the function of these genes remains unclear, bioinformatic data indicates that OtcX3, DpsD and their known homologues have similar conserved catalytic domains and are thus likely to be functionally related.

The Type II R1128 cluster of *Streptomyces* R1128 contains a homologue of *otcX3* (Tang *et al*., 2004). This strain synthesises 4 polyketide anthraquinone compounds, R1128A-D, the structures of which differ depending on the starter unit used (Hori *et al*., 1993a; 1993b). The gene, *zhuC*, encodes a protein that, like OtcX3, was initially assigned as a putative acyltransferase responsible for loading a unique, non-malonyl-CoA-derived starter unit (Meadows and Khosla, 2001). Decarboxylative condensation occurs between an acetyl-, propionyl-, isobutyryl-, or butyryl-CoA derived primer unit and a malonyl-CoA extender unit to yield an acetoacetyl-, β-ketopentanoyl-, 3-oxo-4-methylpentanoyl-, or β-ketohexanoyl-ACP starter unit, either of which are primed followed by condensation of malonyl extender units to form the backbone of the R1128 compounds (Meadows and Khosla, 2001).

The phylogenetic relationship of ZhuC to OtcX3 and other homologous proteins from other Type II PKS was illustrated in the bioinformatic data in **Section 3.1.2**. The most homologous proteins to ZhuC are from Type II PKS' that incorporate a non-acetate starter unit; AknF from the aclacinomycin A/aklavinone cluster of *S. galilaeus* (propionyl starter unit, Raty *et al*., 2002), CosF from the cosmomycin

cluster of *S. olindensis* (propionyl starter unit, Garrido *et al*., 2006), OtcX3 (malonamyl starter unit, Zhang *et al*., 2006), DpsD from the daunorubicin/doxorubicin cluster of *S. peucetius* (propionyl starter unit, Otten *et al*., 1990), FrnK from the frenolicin cluster of *S. roseofulvus* (butyryl starter unit, Bibb *et al*., 1994b) and EncL from the enterocin cluster of *S. maritimus* (benzoyl starter unit, Piel *et al*., 2000). The true function of each protein has remained unclear. However, ZhuC was characterized as an acyl-ACP:thioesterase that prevents the incorporation of unwanted acetyl primer units into the polyketide backbone of the R1128 compounds (Tang *et al*., 2004).

In previous studies, it was discovered that the tetracenomycin (*tcm*) and actinorhodin minimal PKS' could accept complex, non-acetate-derived starter units in the presence of the R1128 initiation module, consisting of *zhuC*, *zhuG* (ACP) and *zhuH* (ketosynthase III) (Marti *et al*., 2000; Meadows and Khosla, 2001; Tang *et al*., 2003; Tang *et al*., 2004b). Polyketides primed with pentanoyl, hexanoyl, or isohexanoyl primer units could be synthesized by *S. coelicolor* CH999 when the minimal PKSs from the *act* and *tcm* (tetracenomycin) clusters and the R1128 initiation module were co-expressed. ZhuC had been shown to have a far inferior binding affinity for malonyl-CoA compared to the malonyl-CoA:ACP acyltransferase (MAT) from the fatty acid synthase of the host, therefore the latter was thought to be sufficient for malonylation of the ACP during the elongation of the polyketide chains after priming of the starter units (Tang *et al*., 2003). However, expression of *zhuC* was necessary for the *in vivo* biosynthesis of these hybrid polyketides, indicating that ZhuC had an essential function in 'communication' between the R1128 initiation module and the minimal PKS' (Tang *et al*., 2004b).

The catalytic properties of ZhuC were studied *in vitro* as an extension of these assays using the *tcm* minimal PKS. Priming the *tcm* initiation module with malonyl-CoA or malonyl-ACP mainly yielded the decaketides SEK15 and SEK15b, while using hexanoyl-CoA or hexanoyl-ACP produced hexanoyl-primed analogues of the octaketides, SEK4 and SEK4b at equimolar concentrations (**Fig 1.14a)** (Fu *et al*.,

1994a; Tang *et al*., 2004). Addition of purified ZhuC to the reaction reduced the activity of the *tcm* PKS by 3-fold in the absence of an acyl donor. The presence of ZhuC and malonyl-CoA abolished biosynthesis of the SEK15/15b decaketides but in the presence of hexanoyl-CoA, the quantity of hex-SEK4/4b octaketides increased dramatically.

It was subsequently demonstrated that ZhuC displayed a 200- and 120-fold greater specificity for binding acetyl-ACP and propionyl-ACP (but not malonyl-ACP), respectively in comparison to hexanoyl- and octanoyl-ACP. This confirmed that ZhuC operated as an 'editing' enzyme; a thioesterase that binds and prevents unwanted acyl units from binding to the starter module, thus allowing the direct priming of the R1128 minimal PKS with alkylacyl units. The low affinity of ZhuC to bind malonyl-ACP made teleological sense, as it would not interfere with subsequent decarboxylative condensation of malonyl extender units during chain elongation (Tang *et al*., 2004). Therefore, it is plausible to consider the possibility of similar 'gatekeeper' roles for OtcX3, DpsY and homologues in the context of OTC and dauno/doxorubicin biosynthesis, respectively.

Potential characterization of *otcX3* using an equivalent assay to that described for ZhuC above would require soluble, active purified recombinant OtcX3, which was not achieved in this work. Empirical evidence indicated that OtcX3 was insoluble and unstable in *E. coli* as described in **Sections 3.2/3.3**, suggesting that homologous expression in a *Streptomyces* host would be a more feasible route towards isolation of stable recombinant OtcX3. The progressive work published by Zhang *et al*., (2006; 2006b; 2007; 2008) and Wang *et al*., (2009) identified the essential genes required for OTC biosynthesis. The work also indicated that a combination of *in vivo* studies of mutants combined with reconstitution assays will be required to fully characterize the remaining genes of the *otc* cluster, including *otcX3*, whose function remains putative or unclear. The data indicates that each of these genes is not essential for the production of a bioactive product but may have more ancillary roles

to maintain the efficiency of the PKS system itself. Other than *otcX3*, these genes include *otcY2-3*, *otcD3* and *otcD5* (**Section 1.7**).

Complementation to the disruption of *otcX3* must be confirmed to complete the *in vivo* studies of *otcX3* described in this work. HPLC analysis must also be expanded to explore and identify any significant differences between the metabolic extracts of the mutant and the wild-type.

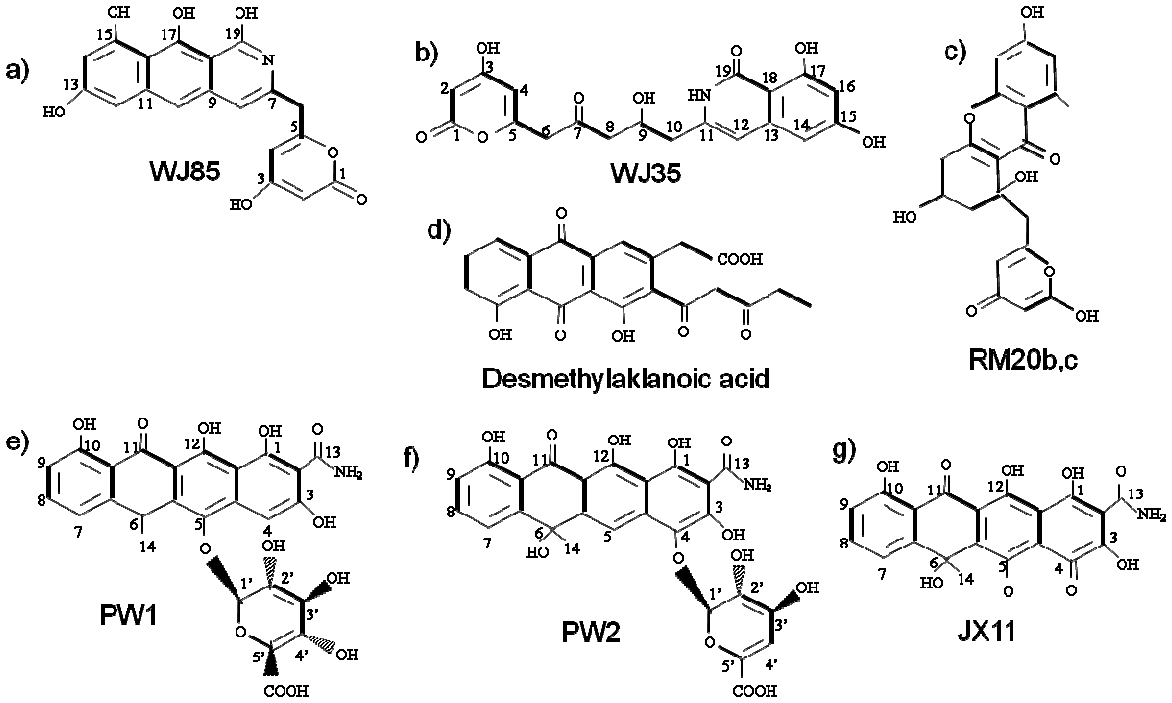


Fig 5.1 **Chemical structures of key novel compounds isolated from analysis of various *otc* genes by heterologous expression in *S. coelicolor* CH999 or *in vivo* disruption in *S. rimosus***

a) WJ85 – *otcY1-1, 1-2, 1-3 +1-4* (pWJ85, Zhang *et al*., 2006b)

b) WJ35 – pWJ85 + *otcY2-1* (pWJ35, Zhang *et al*., 2006)

c) RM20b,c – *otcY1-1, 1-2, 1-3* + *actIII* (pSEK20, Fu *et al*., 1994b)

d) Desmethylaklanoic acid: pWJ119 – *otcY1-4* (Zhang *et al*., 2007)

e) PW1 – produced by Δ*otcY1-5* mutant (Wang *et al*., 2009)

f) PW2 – produced by Δ*otcD2* mutant (Wang *et al*., 2009)

g) JX11 – pWJ119 + *otcY1-5* (Wang *et al*., 2009)

See text for further details.

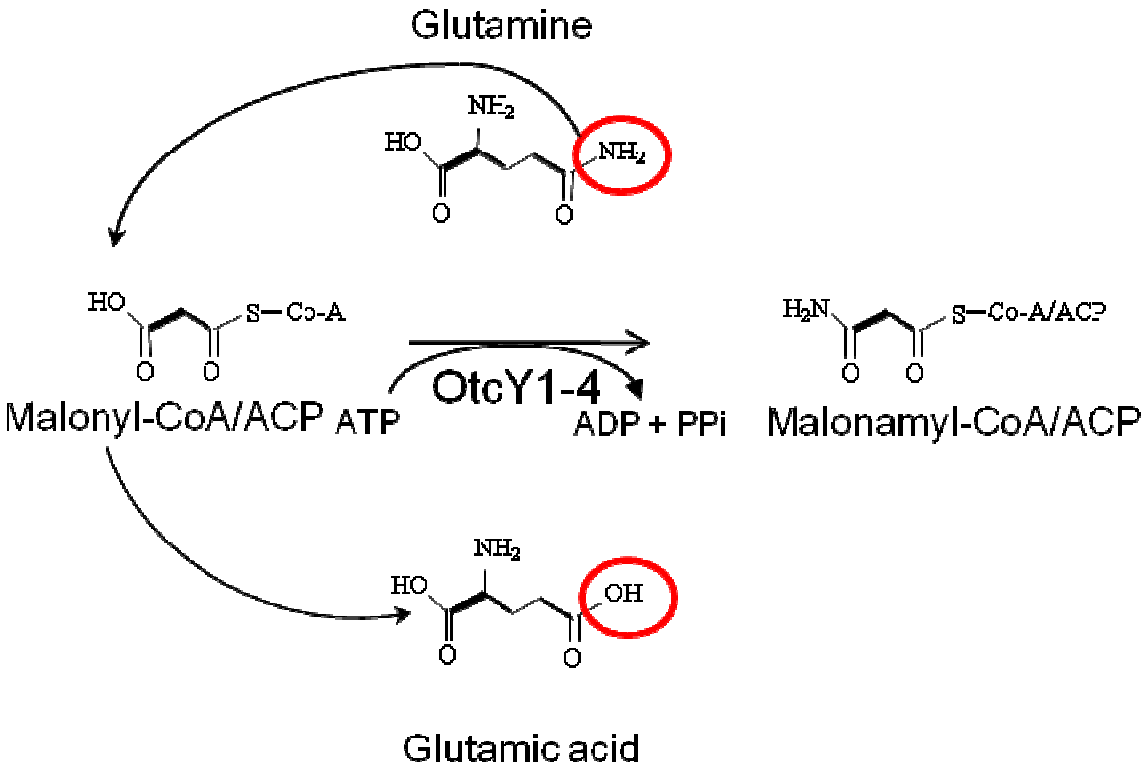
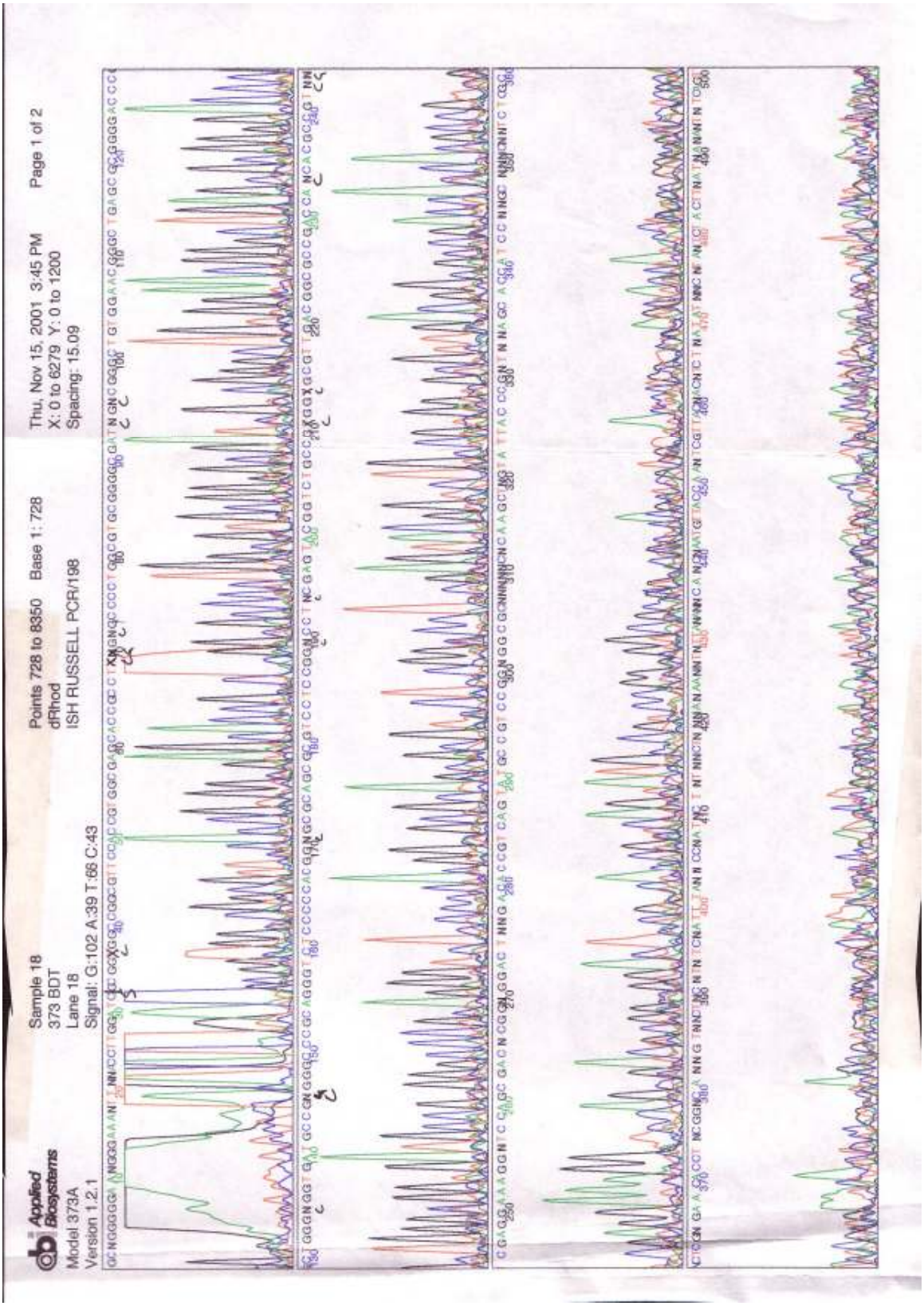


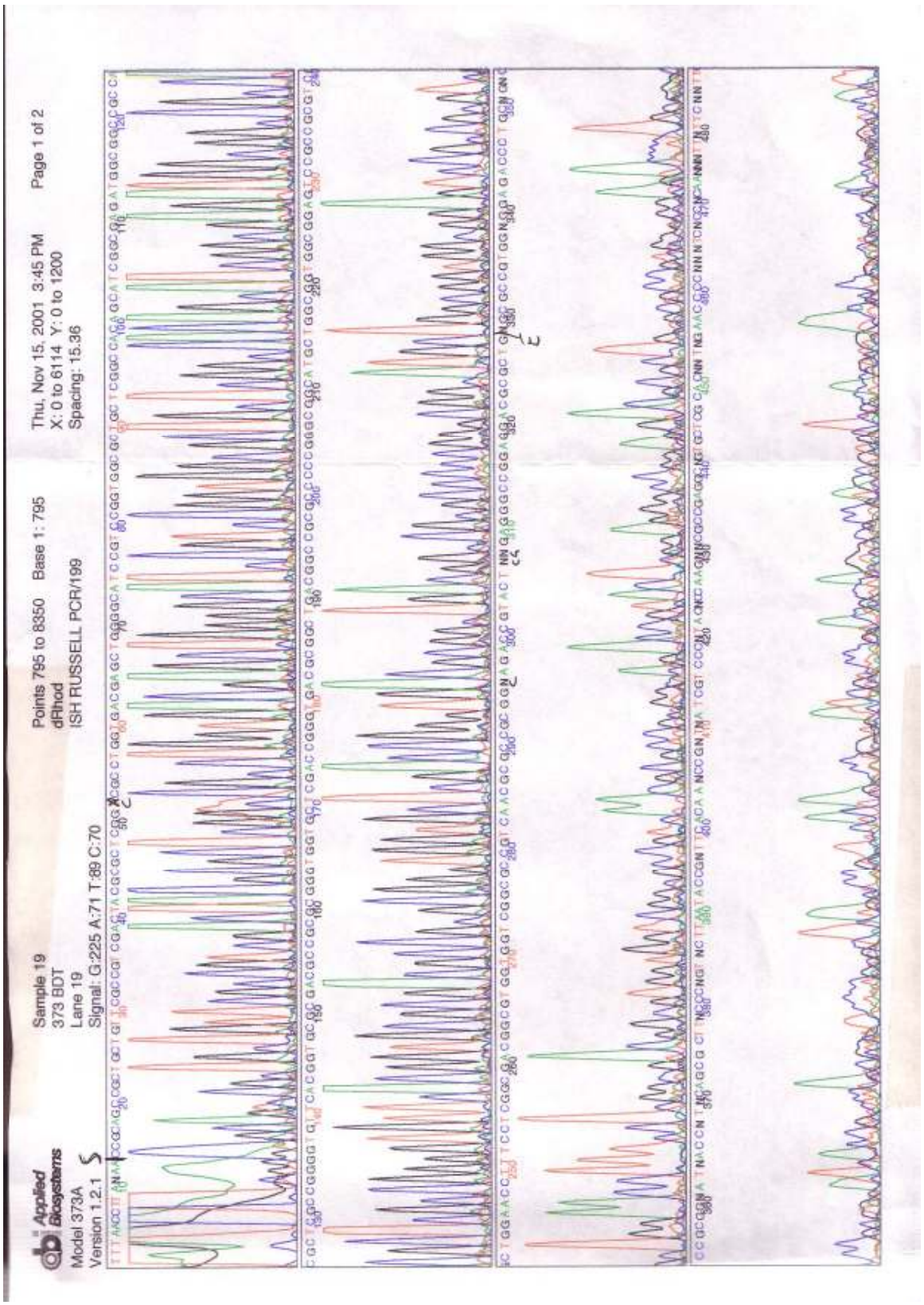
Fig 5.2 **Hypothesized model for the biosynthesis of the malonamyl priming unit in OTC biosynthesis by *otcY1-4***

As proposed by Zhang *et al*., (2007). See text for details.

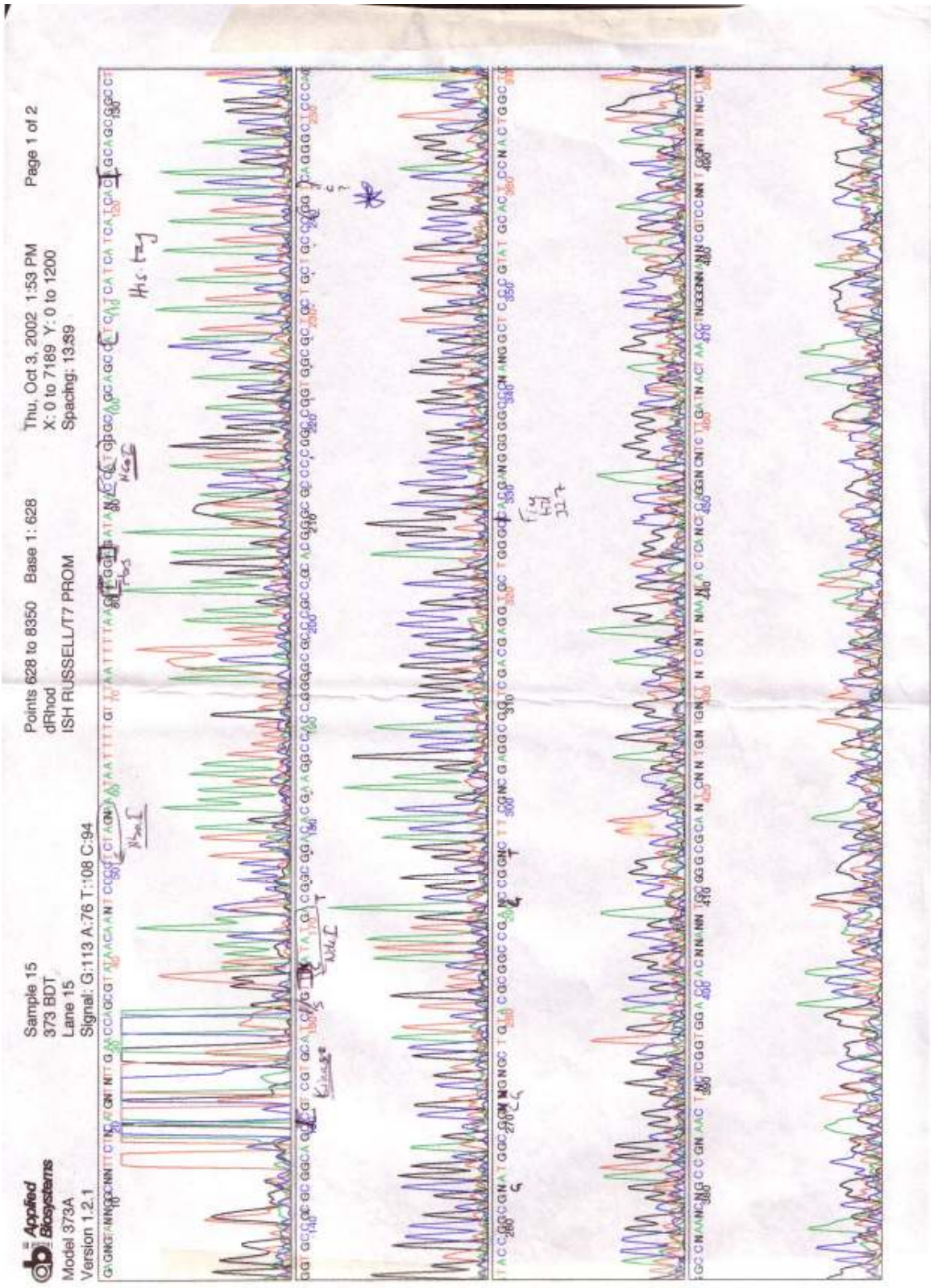
Appendix



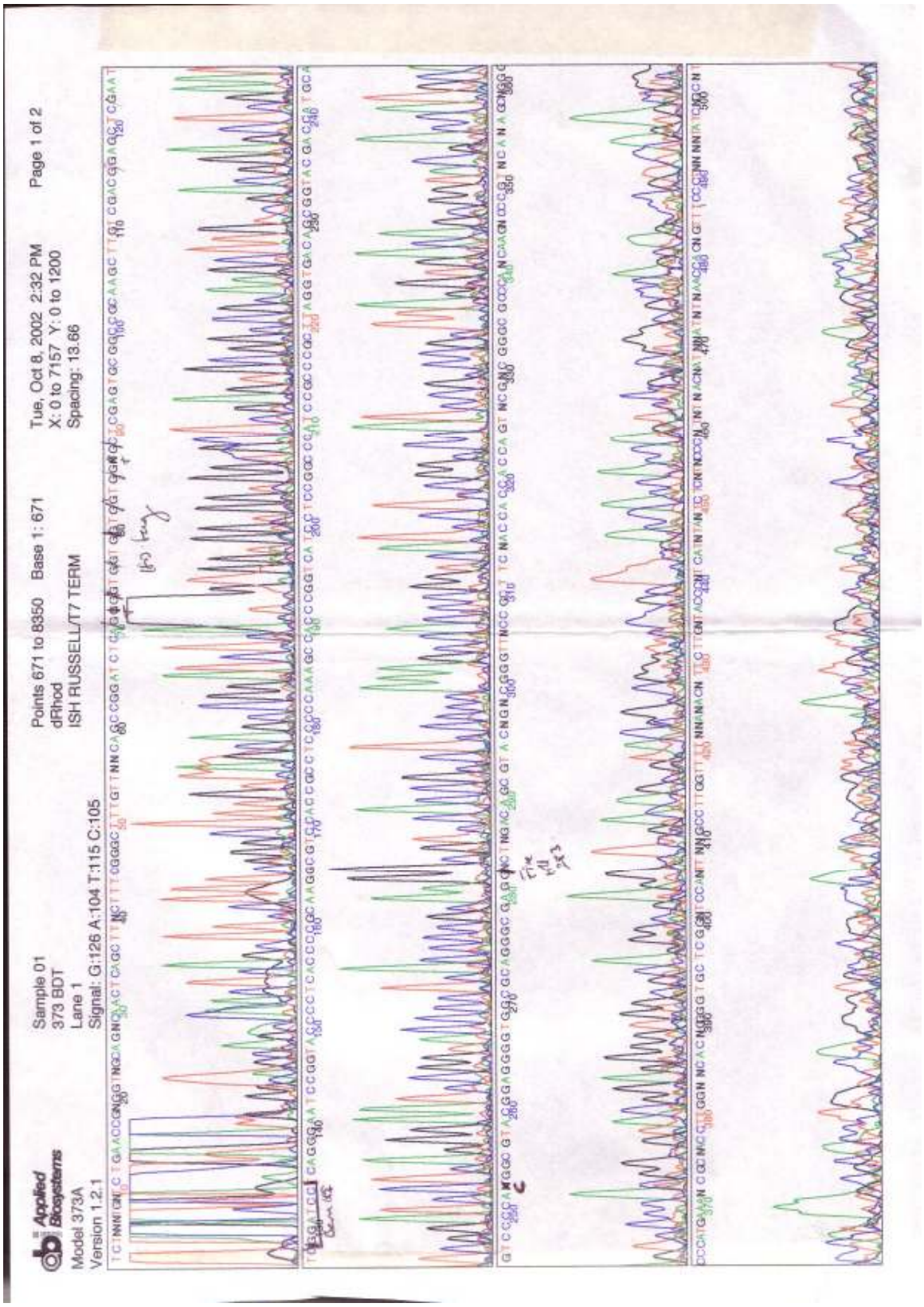
A. 1 Sequence data of *otc*X3 PCR product synthesised with Red Taq Polymerase (sense strand, Section 3.1.4).



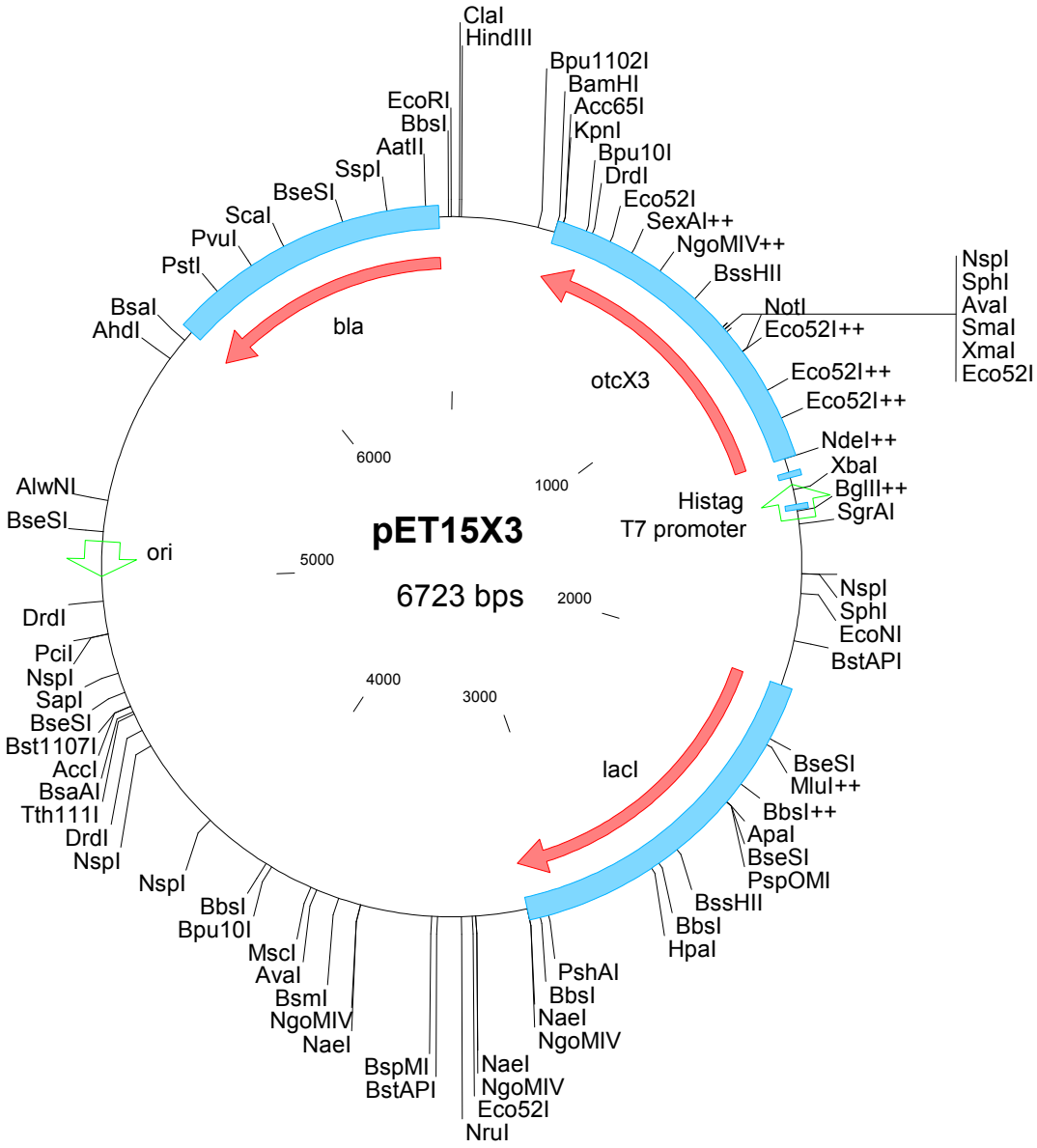
A. 2 Sequence data of *otc*X3 PCR product synthesised with Red Taq Polymerase (anti-sense strand, Section 3.1.4).



A. 3 Sequence data of *otc*X3 PCR product cloned into pET33b(+), synthesised with Red Taq Polymerase (sense strand, Section 3.1.4).

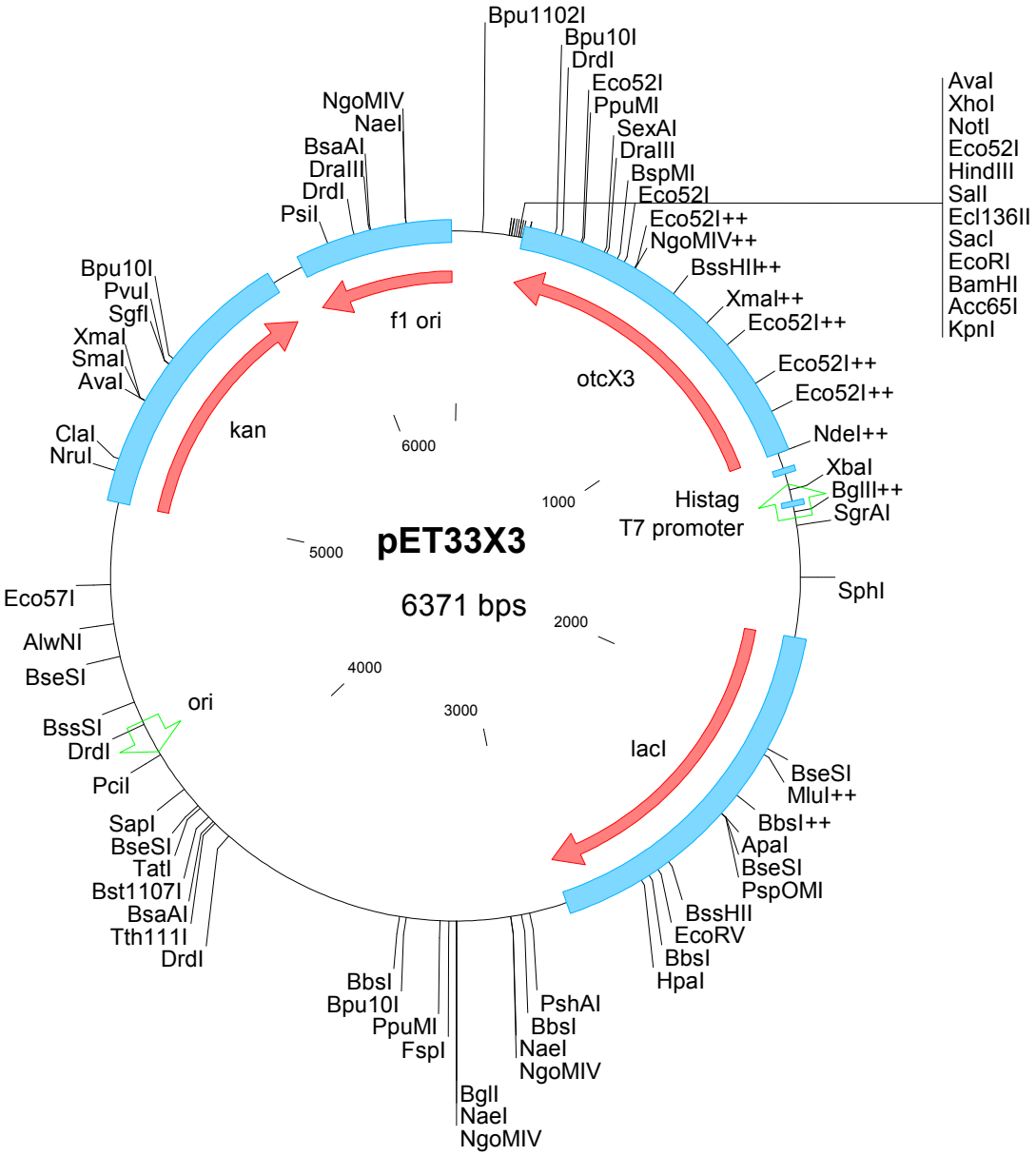


A. 4 Sequence data of *otc*X3 PCR product cloned into pET33b(+), synthesised with Red Taq Polymerase (anti-sense strand, Section 3.1.4).



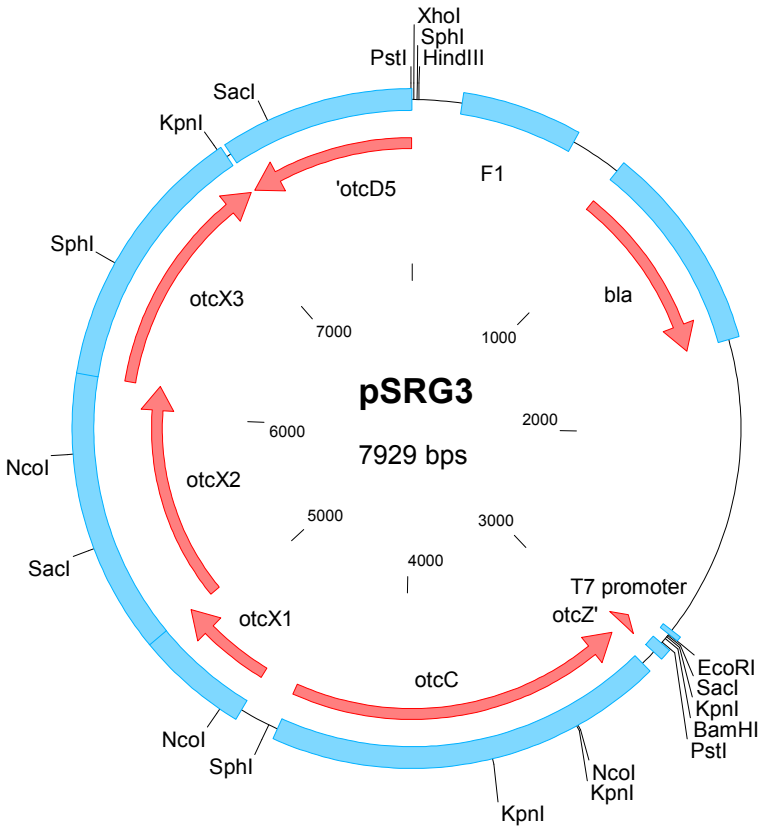
A. 5 Map of pET15X3

1.026kb *Nde*I/*Bam*HI *otc*X3 cassette, ligated into pET15b (www.novagen.com) (**Section 3.1.4**).



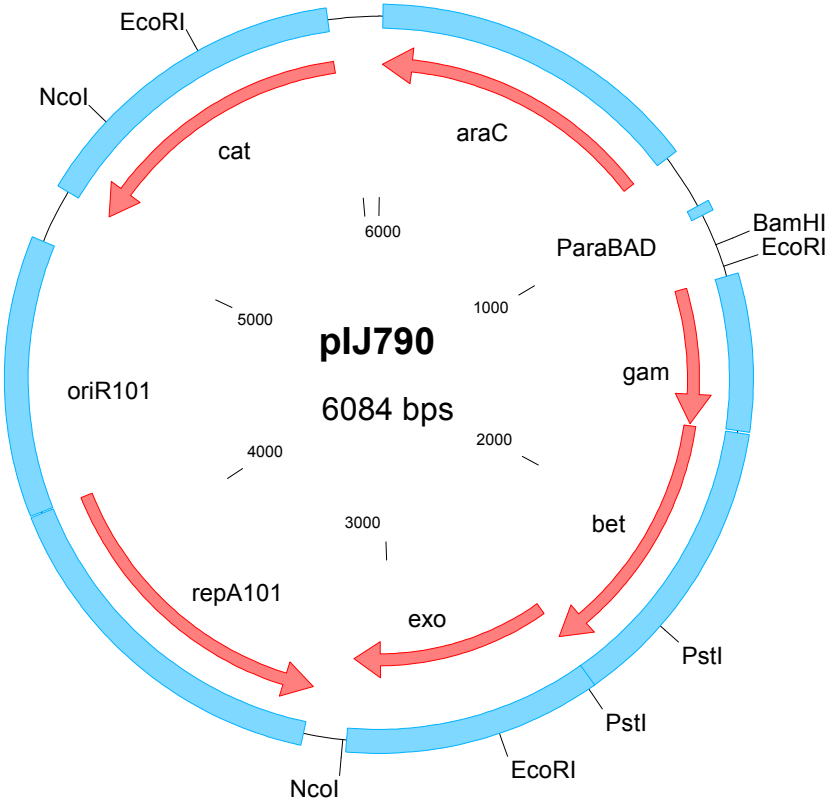
A. 6 Map of pET33X3

1.026kb *Nde*I/*Bam*HI *otc*X3 cassette, ligated into pET33b(+) (www.novagen.com) (**Section 3.1.4**).



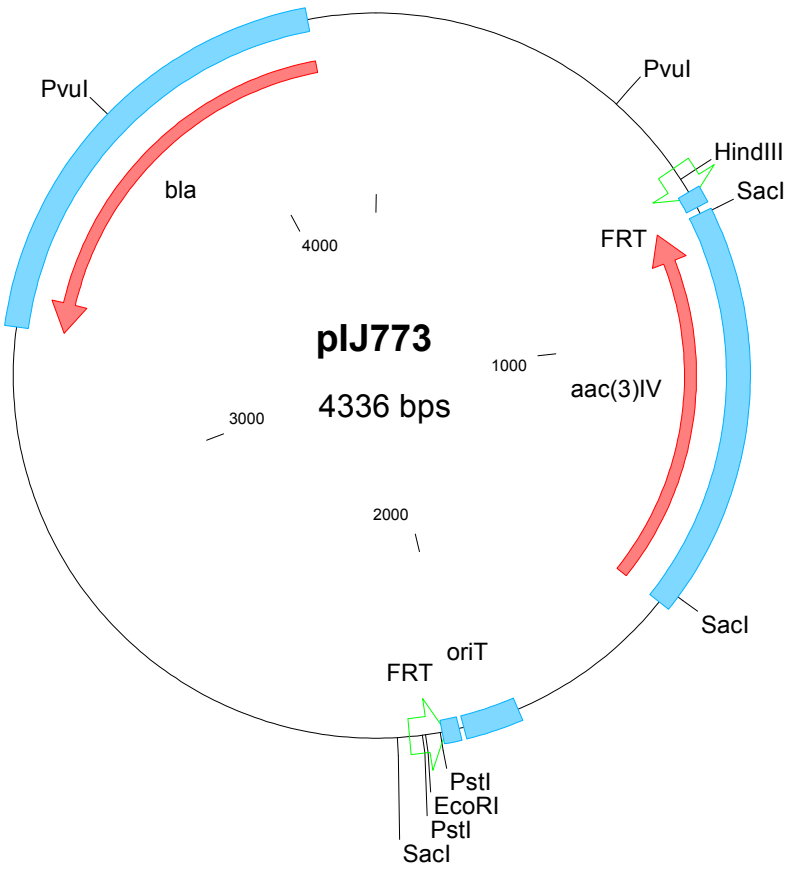
A 7. Map of pSRG3 (Garven, 1995)

Used as a template to amplify *otcX3* in PCR in **Section 3.1.3** and 5.2kb *Pst*I fragment used as a probe for Southern blots in **Section 4.3**. 5.2kb *Pst*I fragment from *otc* cluster into pIBI24 (Dente *et al*., 1983)



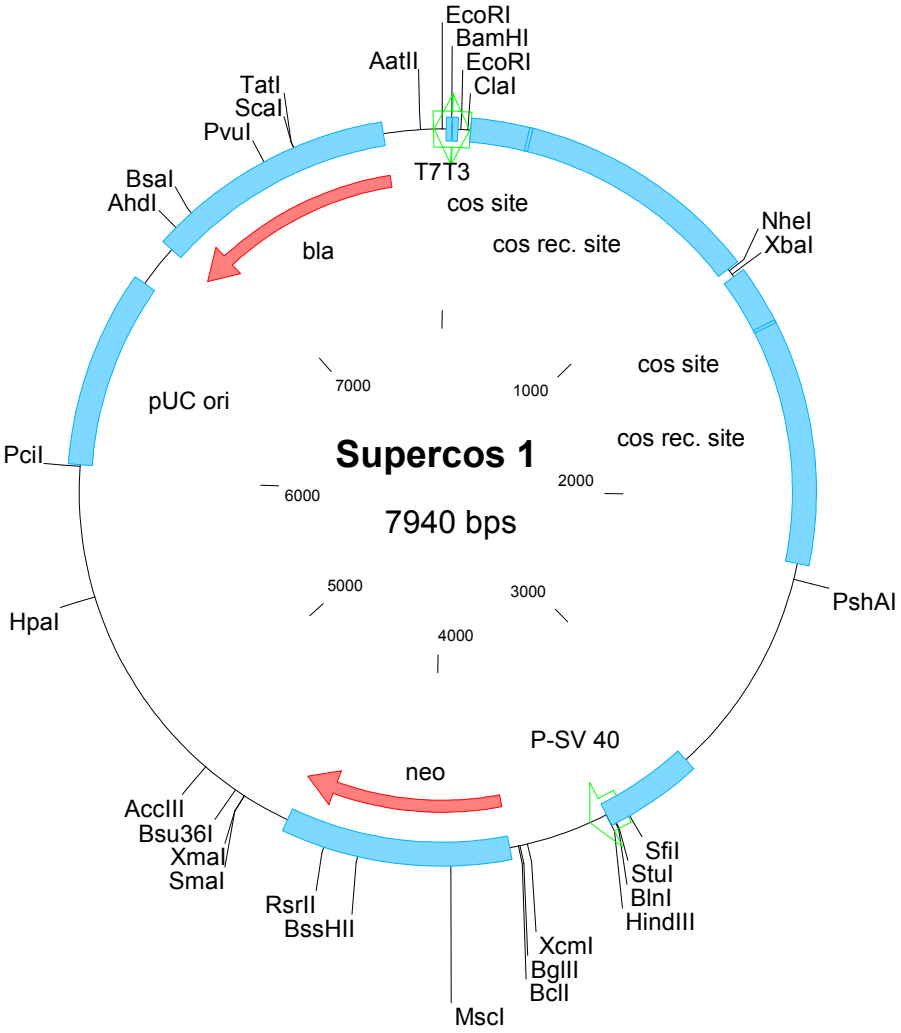
A. 8 Map of pIJ790

Derived from pKD20 (Datsenko and Wanner, 2000). Ampicillin resistance gene, *bla*, replaced by choramphenicol resistance gene, *cat*, to allow selection with Supercos 1 in *E. coli*. Used in REdirect© protocol (**Section 4.3**) (Gust *et al*., 2002).



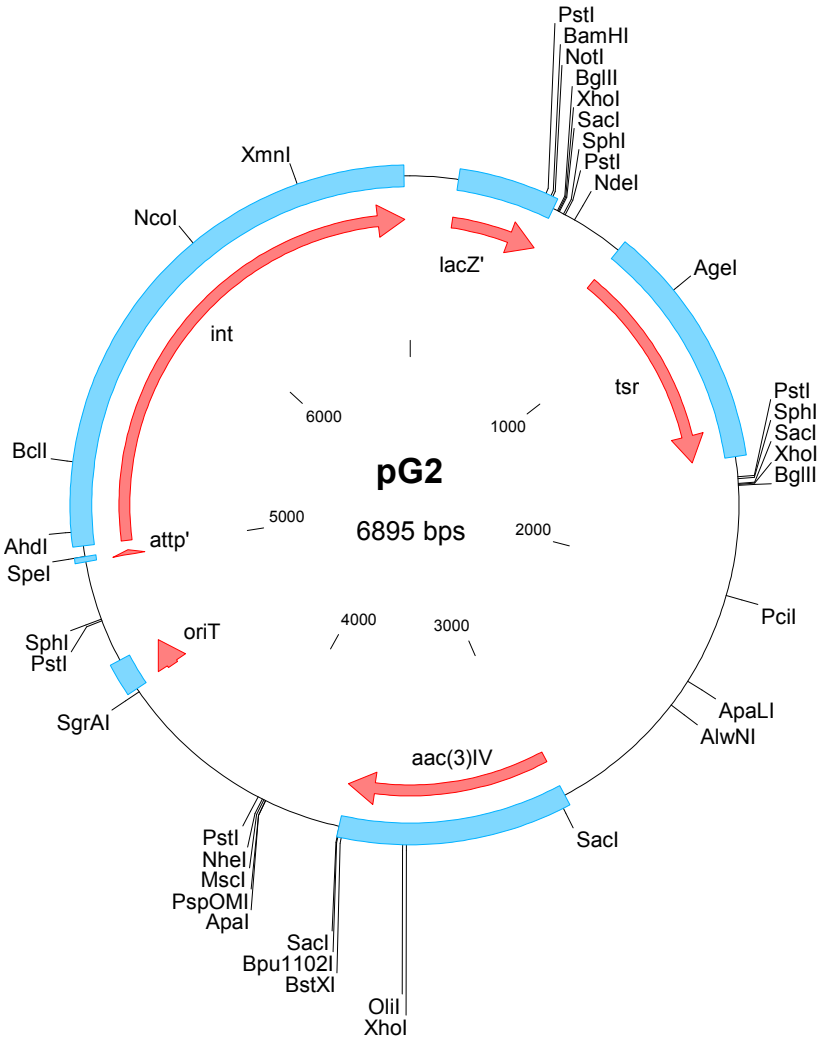
A. 9 Map of pIJ773

Contains the *aac(3)*IV-*oriT* cassette used in REdirect© protocol (Gust *et al*., 2002) (**Section 4.3**).



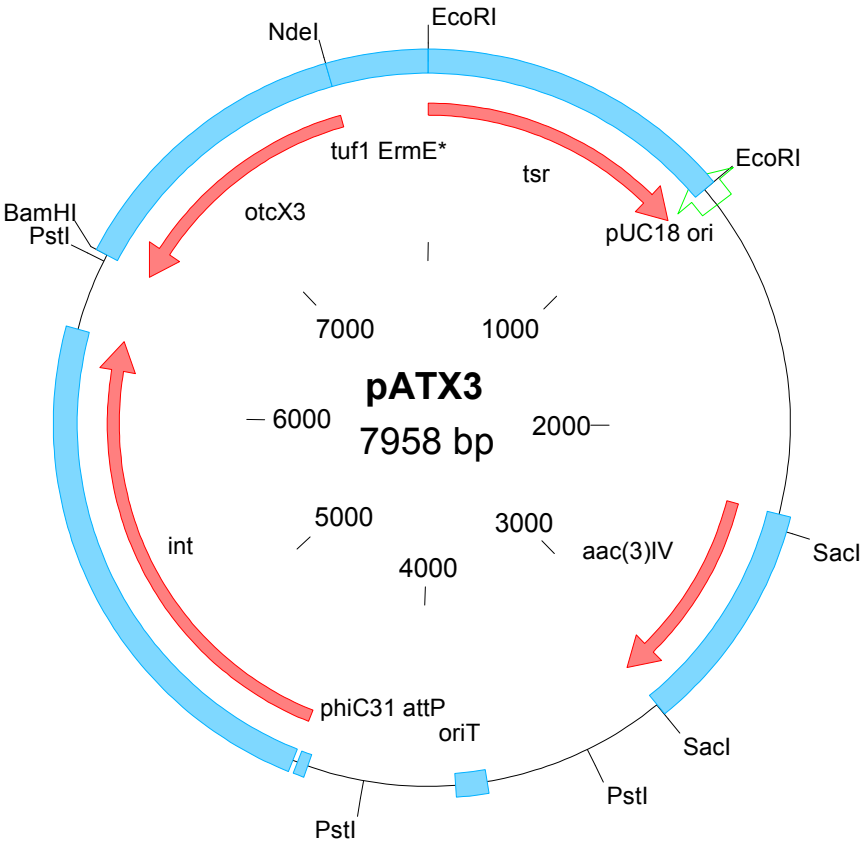
A. 10 Map of Supercos 1

Used for the construction of *S. rimosus* M4018 cosmid library (Almuteirie, personal comm.) from which, cosmids AH6 and EH1 were isolated, www.stratagene.com.



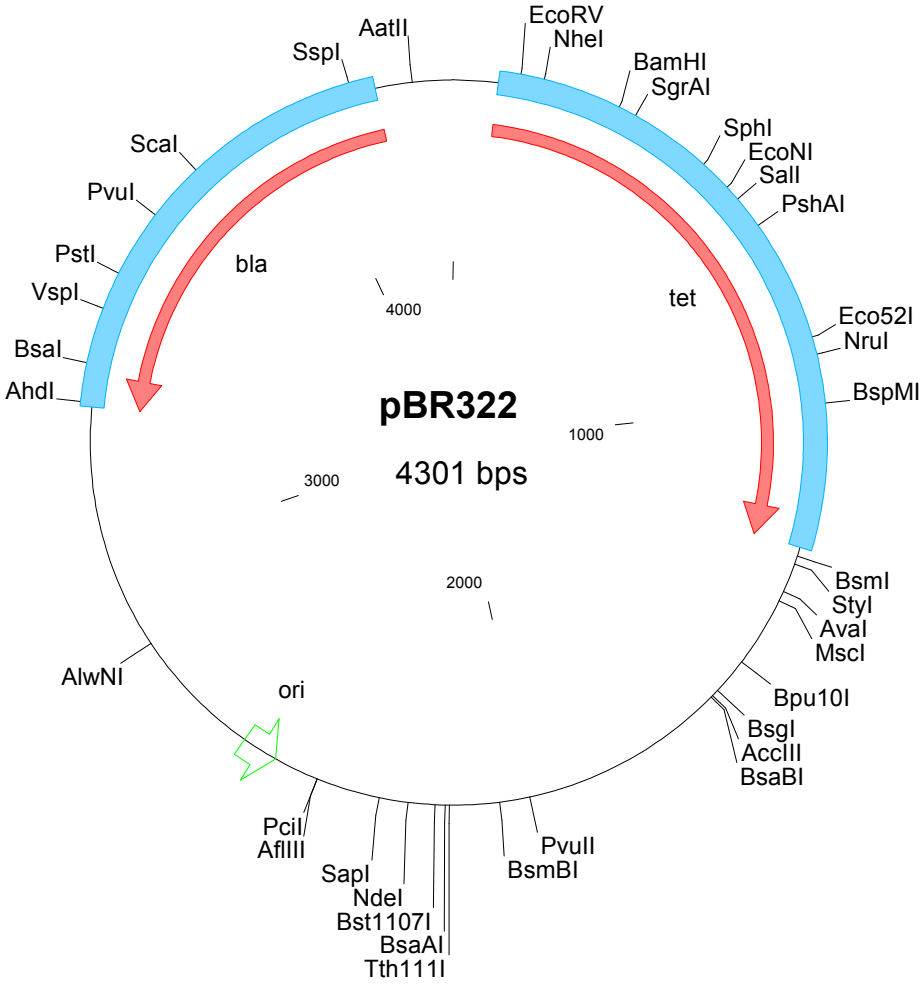
A. 11 Map of pG2

E. coli/Streptomyces shuttle vector. Encodes thiostrepton and apramycin resistance. Used as a control to transform *S. rimosus* M4018 during REdirect protocol (**Section 4.2**, Almuteirie, Personal comm.).



A. 12 Map of pATX3

Integration/expression vector. Encodes thiostrepton and apramycin resistance. Used to attempt complementation of putative mutant disrupted at *otcX3* (**Section 4.3.7**, Tilley, personal comm.).



A. 13 Map of pBR322

Encodes tetracycline and ampicillin resistance. Used as a control in bioassay of disrupted mutants (**Section 4.3.9**, Sutcliffe, 1979).

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